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CRYSTAL GROWTH IN MINERALIZING TISSUES*

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SUMMARY

The growth of nuclei into crystals requires a metastable solution phase with respect to the growing particles (supersaturation), and the presence of impurities. The rate of growth is proportional to the diameter of the particles, and is related to the surface to volume ratio of the crystal. Trace components can modify crystal habit and may be factors in determining the characteristics of bone mineral. Orientation of bone crystals parallel to the collagen fibers implies that the organic matrix acts as a habit modifier. The small size of bone hydroxyapatite crystals (640 Å or less), in contrast to the synthetic hydroxyapatite crystals prepared in the laboratory ($2 \cdot 10^7$ Å or 2.0 mm), suggests that factors *in vivo* operate to limit their size. Cessation of crystal growth in a supersaturated solution is likely to be caused by surface adsorption of inhibiting ions or compounds. A mechanical barrier imposed by the collagen fibrils, and decreased renewal of the fluids surrounding the crystal may also be involved. The inorganic portion of mineralized tissue is in two phases: a crystalline core of hydroxyapatite of $[Ca_{10}(PO_4)_6(OH)_2]$ and an adsorbed outer shell which is of an amorphous or gel-like nature, containing anions such as carbonate, citrate, fluorides, and cations such as Na, Mg, Pb, and Sr. This does not preclude entry of a few such ions into the lattice. The inorganic composition of bones and teeth depends on the composition of the surrounding fluids (which can be regulated by dietary constituents). For example, the CO_3/PO_4 ratio of minerals in bone and teeth is related to the CO_3/PO_4 ratio of blood serum.

INTRODUCTION

The formation of nuclei for the growth of bone crystal has been dealt with at length in the preceding paper[1]. This communication is devoted to the development of the nuclei into hydroxyapatite crystals, factors determining the shape and position of the crystals, and the forces limiting the size of the crystals and causing cessation of crystal growth.

The inorganic fraction of bone (65–70 % by weight) consists of crystals which are generally oriented with respect to the organic matrix (30–35 % of the bone, predominantly (approximately 95 %) collagen[2]). This orientation suggests that the deposition of crystals (a three stage process of nucleation, crystal growth, and cessation of crystal growth) is modified by factors within the matrix. For this reason the discussion includes not only the humoral factors, but also the possible influence of the matrix in the development of the crystal.

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Crystal growth

The growth of nuclei into ionic crystals, and the further growth of the crystals, depend on several factors. One such factor is the maintenance of a state of supersaturation (metastable liquid phase) with respect to the nuclei. A second factor, according to TURKEVITCH, is the presence of an impurity in order that nuclei may grow into larger particles*[3]. To possess a "driving force" for further growth, fine particles must have physical or chemical imperfections. This is in harmony with FRANK's concept of spiral dislocation, a self-perpetuating surface imperfection, which permits the addition of ions layer by layer[4].

Regarding the rate of growth of bone hydroxyapatite crystals, which are of colloidal dimensions, it is interesting to consider the law of growth of fine particles, discussed by TURKEVITCH[3]. The rate of growth of a colloidal particle is proportional to its diameter. That is, the larger the particle the faster it grows, and the smaller the particle the slower it grows. This also follows from the equations developed by BEST[5] for the solubility product of calcium phosphate (equation 1):

$$(\mathrm{Ca})_e^{\nu_c}\,(\mathrm{PO_4})_e^{\nu_p} = \mathrm{e}^{\frac{\mu_i - \mu_0'}{RT}}\;\mathrm{e}^{\frac{(\mu_s - \mu_i)}{RT}\xi\delta} = K \tag{1}$$

As the crystal becomes larger the value ξ approaches zero, and the equation takes the form of equation 2:

$$K_0 = \mathrm{e}^{\left(\frac{\mu_i - \mu_0'}{RT}\right)} = \lim_{\xi \to 0} K \tag{2}$$

μ_i, chemical potential of interior of crystal; μ_s, chemical potential of surface of crystal; μ_0', chemical potential of supernatant; v_c, number of calcium ions in elementary lattice unit; v_p, number of phosphate ions in elementary lattice unit; R, gas constant; T, absolute temperature; ξ, surface to volume ratio; δ, mean distance between adjacent ions of opposite charge in lattice.

It follows from the above equation that in a certain region of colloidal dimensions larger crystals being less soluble will grow, while smaller crystals go into solution. This is in contrast to the rate of growth of crystals of microscopic size, where the rate is proportional to the surface area[6], which means, in effect, that the rate of growth is inversely proportional to the diameter.

The initial fragments of bone salt are likely to be a simple phosphate such as $CaHPO_4$. This compound, under appropriate conditions, *e.g.*, pH above 6.9, transforms by accretion or recrystallization to the less soluble hydroxyapatite[7]. The growth of hydroxyapatite would be expected to take place more rapidly than that of $CaHPO_4$ because the surrounding fluids are more highly supersaturated with respect to the less soluble hydroxyapatite. Thus, a slow initial growth rate of $CaHPO_4$ (or some other simple calcium phosphate) would give way to a more rapid rate of crystal growth after transformation of the initial solid phase to hydroxyapatite. The increased velocity of crystal growth after transformation and with increasing diameter of the particles may explain why, by the time the developing osteons are discernable, they are already at least 70 % mineralized (see ref. [7], p. 107 and ref. [8]), and may account, too, for the extremely rapid rate of calcification *in vitro*[9]. In addition, the above mode of crystal growth is supported by observations that the deposition of mineral on reconstituted collagen fibers is extremely slow during the first 24 h of incubation, in contrast to an extremely rapid rate of mineralization in the second 24 h[10].

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* To test this concept for bone hydroxyapatite it would be necessary to prepare highly purified reagents, as the ones normally used to make up calcifying solution contain small traces of impurities. The effects of known added impurities could then be ascertained.

Habit modification

The character of the final bone crystal depends on the basic lattice structure, which will be discussed below, as well as the influence of trace impurities in modifying the shape of the growing crystal. The action of such impurities, referred to as habit modifiers, is illustrated in Fig. 1[11]. Habit modification has been demonstrated to occur in hydroxyapatite on exposure to various concentrations of citrate, which affected both the size and the shape of the crystals[12]. Dyes which can be preferentially adsorbed to one facet of a crystal can modify its growth so that it takes place more rapidly on other planes[13]. As a simple example, if growth of the crystal is uninhibited, then all facets will grow at their normal rate (Fig. 2a, b). If, however, two of three dimensions

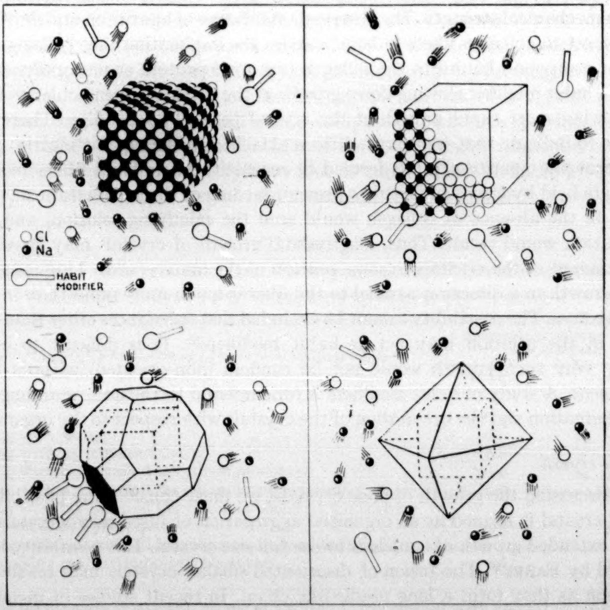

Fig. 1. Stages in the modification of habit of sodium chloride crystal from cubic to octahedral.

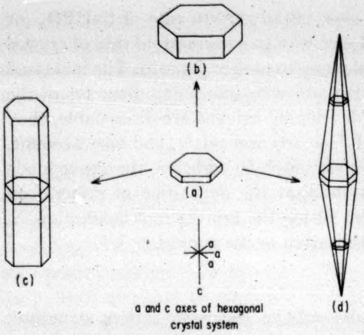

Fig. 2. Habit modification of hexagonal crystal (schematic). (a) Original hexagonal crystal. (b) Hexagonal crystal after further growth with no habit modification. (c) Habit modification with inhibition of growth along a-axes, resulting in elongation along c-axis. This could be caused by preferential adsorption of an inhibitor on the rectangular planes or by a mechanical barrier along these planes. (d) Habit modification with convergence of a-axes to a point, while the crystal elongates in its c-axis. Such habit modification would result from surface inhibition of the initially rectangular planes, which continues to be applied, while additional material is being deposited on the hexagonal ends. Here the hexagonal dimensions decrease with continued inhibition, until a point (or zero dimensions) is reached, and crystal growth stops.

are inhibited from further growth, then the inhibited plane will remain small, and the third dimension will elongate. The growing crystal, being confined as in a "straight jacket", will enlarge in the long axis (Fig. 2 a, c). Inhibiting forces applied continuously can cause convergence of the elongating crystal to a point, *i.e.*, the dimensions of the facets in the inhibited plane will gradually decrease to zero. Crystal growth will then cease (Fig. 2 a, d).

In bones and teeth, habit modifiers (or conceivably mechanical barriers) appear to be arranged favorably for the longitudinal growth of crystals in the direction of greatest mechanical strength. The observed orientation of apatite crystals[14–16], parallel with respect to collagen fibers in bone, carries the implication that collagen or some group or compound bound to the collagen (*e.g.* mucoprotein or mucopolysaccharide) may be a habit modifier slowing down growth of the crystal perpendicular to the fiber, and allowing more rapid growth of the crystal parallel to the fiber. There is some evidence to indicate that hydroxyapatite is attached to the organic matrix, as it was shown that the apatite crystals formed in reconstituted collagen fibers mineralized *in vitro* are held by forces sufficient to prevent seeding of the supernatant fluid[17]. Such crystals in the absence of collagen would seed the calcifying solution, and random precipitation would result. Thus, longitudinal growth of crystals may be visualized by attachment of the crystals to some portion of the matrix, or by long range forces, so that growth in a direction parallel to the fiber is much more rapid than in a transverse direction. The possibility cannot be excluded that substances other than collagen present in the solution may act as habit modifiers[18]. It is difficult to establish, however, why such growth would not be random (non-oriented) without collagen involvement. A study of habit modifiers is fundamental to the understanding of bone crystal formation and the orientation of the crystals with respect to the organic fibers.

The final crystal

In discussing the growth of bone crystals, we must consider the possibility that the final crystal is formed as an organized aggregation of fused microcrystals, rather than by extended growth of a nucleus to the full size crystal. This possibility has been suggested by Fabry[19]. The fusion of disoriented smaller crystals may result in their orientation as they form a long needle-like chain. In recent studies of membranous (parietal) bone of one week old mice by electron microscopy[20], Molnar observed calcification in collagen fibers which were non-striated, and apparently the mineral deposited there was amorphous or consisted of crystals smaller than 15 Å, without evident orientation with respect to the organic fibers. In a deeper layer she observed collagen with cross-striations of a periodicity of 510–550 Å, where calcification occurred in all the sub-bands in the form of small crystals 20–30 × 50 Å, aligned parallel to the collagen fibrils. These tiny crystals later seem to meet each other to form a continuum of crystals of the dimensions 35–60 × 125–800 Å, implying that a series of nuclei of crystal growth, produced in the matrix, ultimately form one continuous needle-like solid from the union of the smaller crystals.

Cessation of crystal growth

Present knowledge suggests that intrinsic factors within the bone hydroxyapatite crystals operate to limit their size. The largest hydroxyapatite crystal that has been prepared in the laboratory is 2.0 mm, or $2 \cdot 10^7$ Å, in contrast to other crystals which can be grown to huge dimensions. However, at present no satisfactory theory can be presented to predict the maximum size to which a crystal will grow. The factors limiting the size of bone or tooth hydroxyapatite crystals *in vivo* appear to be in effect well before they attain the size of 2.0 mm. CARLSTRÖM AND FINEAN[15] reported a crystal 220 Å long. More recently, CARLSTRÖM AND GLAS[22] reported that the crystal is 640 Å long. Enamel apatite crystals as long as 1,600 to 6,000 Å have been observed[16, 23, 24]. The possibility has been proposed, however, that the larger enamel crystal is composed of smaller units, about 300 Å long, held together by organic fibers[25]. Habit modification is a probable contributor to the small crystal size.

Two general concepts of the mechanism of cessation of crystal growth in a supersaturated solution have become the subjects of speculation by investigators.

1. Certain ions or compounds adsorbed on the surface of a crystal in bonds stronger than those exerted among the ions composing the lattice may interfere with crystal growth[13, 26–28]. Experimental evidence for the reversible inhibition of crystal growth by surface binding of beryllium ions is summarized in Table I[27, 28]. When $Ca_3(PO_4)_2$ was shaken with 100 ml of 0.1 mmole/l Be^{++}, whether in water or in 75

TABLE I

REVERSIBLE INHIBITION OF CRYSTAL GROWTH OF TERTIARY CALCIUM PHOSPHATE

(Incubation at Ca(10 mg %) × P(4.8 mg %) = 48 at pH 7.3 for 18 h.

*Treatment of $Ca_3(PO_4)_2$ before incubating 2.5 mg in 50 ml calcifying solution**	*Concentration of calcifying solution after incubation*	
	Ca, mg %	*P, mg %*
None	6.0	3.0
1 h shaking with water	6.7	3.3
1 h shaking with 75 mmoles/l $CaCl_2$	6.8	3.3
1 h shaking with 0.1 mmole/l beryllium in water	10.0	4.8
1 h shaking with 0.1 mmole/l beryllium in 75 mmoles/l $CaCl_2$	9.8	4.7
1 h shaking with 0.1 mmole/l beryllium in water; 0.5 h shaking with 50 mmoles/l phosphate	9.8	4.7
1 h shaking with 0.1 mmole/l beryllium in 75 mmoles/l $CaCl_2$; 0.5 h shaking with 50 mmoles/l phosphate	7.6	3.9

* In the preliminary treatment, 10 mg of $Ca_3(PO_4)_2$ was treated with 100 ml of the indicated solution(s) followed by washing with distilled water and drying at 105°.

mmole/l $CaCl_2$, and then incubated in *in vitro* calcifying solution [Ca (10 mg %) × P (4.8 mg %) = 48], the ability of the tertiary calcium phosphate to remove calcium and phosphate from the solution was inhibited. Calcium phosphate given no preliminary treatment, or shaken in water or calcium chloride solution prior to incubation in calcifying solution, removed calcium and phosphate from the solution during an 18-h incubation period. When $Ca_3(PO_4)_2$ was shaken with beryllium in the presence of $CaCl_2$, its ability to remove calcium and phosphate from the solution was restored upon subsequent treatment with phosphate. On the other hand, calcium phosphate shaken with beryllium in distilled water and then treated with phosphate did not demonstrate this reversal of beryllium inhibition. It is presumed that phosphate treatment binds beryllium so that it is either removed from the crystal surface or does not interfere with crystal growth. Beryllium inhibition in the presence of $CaCl_2$ is reversible, conceivably because the calcium competitively limits the attachment of beryllium at the sites of crystal growth, or influences the type or degree of binding of beryllium to the crystal surface. Analogous behavior was demonstrated for "nucleated" bone sections, *i.e.*, bone sections pretreated with calcium ions followed by phosphate[28].

Both reversible and irreversible inhibition of crystal growth by a surface ion may play a role in the cessation of bone crystal growth. Such ions as carbonate and citrate, or possibly polyphosphates or some organic cation, may be proposed as causing the cessation of crystal growth. Our studies indicate that in the calcification of collagen, as well as in preosseous cartilage, crystals are attached to the organic matrix[17]. Thus, groups in the organic matrix, coming into firmer contact with the larger crystals may be the equivalent of a surface inhibitor. The action of surface inhibitors is discussed earlier with respect to habit modification, and is offered as an explanation of inhibitors which become effective only when the crystals reach a certain size, in contrast to other inhibitors such as beryllium, which will stop crystal growth at a wide range of sizes.

2. A second concept of the cessation of crystal growth, proposed by ROBINSON[29], is that growing bone crystals displace water. A point is reached where the displaced water surrounding the crystal forms a layer so thin that an insufficiency of dissolved minerals reach the crystal. This concept is based on the assumption that the organic matrix becomes less permeable to the extracellular fluids as the crystal matures.

The second mechanism is not likely to work in its simplest form owing to the fact that the crystal is in dynamic equilibrium with the body fluids. As judged by the final anatomical picture, both mechanisms may be visualized as playing a role in the cessation of crystal growth, since the mature inorganic crystal is in direct contact with the collagen fiber without any appreciable water space remaining. IRVING's work with Sudan Black also indicates that the substance stained by the dye (presumably some component of the organic matrix) is associated with the bone crystal at the onset of crystallization[30].

Structure and composition of bones and teeth

The inorganic portion of bone consists of two phases: a crystalline core and an amorphous gel-like outer shell[23]. There is overwhelming evidence that the crystal lattice is made up of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, which may vary in composition from $Ca_6(PO_4)_6(OH)_2^{-8}$ to $Ca_{14}(PO_4)_6(OH)_2^{+8}$. Practically all of the remainder of the mineral content of the calcified tissues is in the amorphous phase, external to the

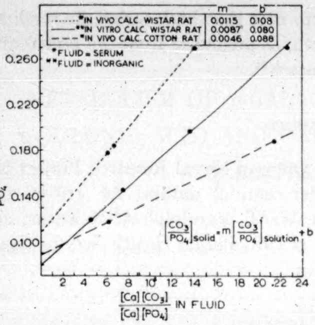

Fig. 3. CO_3/PO_4 ratios of bone mineral calcified *in vivo* and *in vitro* in relation to fluid composition.

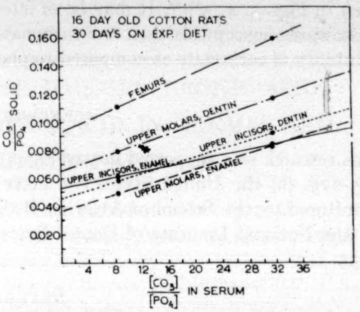

Fig. 4. CO_3/PO_4 ratios of the bones and teeth of the cotton rat in relation to serum composition.

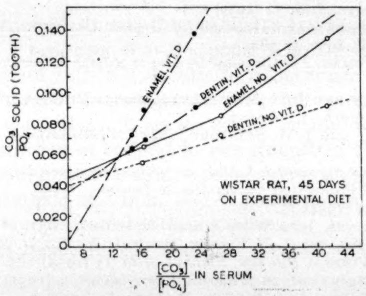

Fig. 5. CO_3/PO_4 ratios of tooth mineral in relation to serum composition in the incisors of the Wistar rat after 45 days on experimental diets.

apatite lattice, although there can be substitutions by fluoride, limited substitutions by lead, and perhaps some strontium ions in the lattice*.

Surrounding the crystalline core, practically all of the carbonate, citrate, and strontium contained in bone mineral are present, tightly adsorbed on the surface of the hydroxyapatite crystal in an amorphous, gel-like, phase. These ions are not incorporated within the lattice structure. Most other ions detectable in bone salt are also likely to be adsorbed. The amount of carbonate adsorbed depends on the CO_3/PO_4 ratio of the solution[32–34], which is a function of the ratio of the activity products $[Ca^{++}]\ [CO_3^{=}]$ to $[Ca^{++}]\ [HPO_4^{=}]$. Similarly, it is likely that strontium deposition, which can be as much as 6.1 %[8], is related to the ratio of the activity products $[Sr^{++}]\ [HPO_4^{=}]$ to $[Ca^{++}]\ [HPO_4^{=}]$ and thus, to the Sr/Ca ratio.

The composition of calcified tissues *in vivo* is related to the composition of blood serum (which can be regulated by dietary calcium, phosphate, and vitamin D), as

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[*] **Posner**, *et al.*[31], proposed that in bone crystal there is an imperfection of the apatite lattice structure in which calcium ions are missing. The apatite phase of the inorganic portion of bones and teeth is of the pseudo-apatite class, also referred to as "defect apatite".

seen in Figs. 3, 4, 5[33,34]. It may be of interest to note that high carbonate teeth are more caries-susceptible than low carbonate teeth, as predicted from the preferential solubility of carbonate as compared to phosphate[33,34].

ACKNOWLEDGEMENTS

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METABOLISM OF D-GALACTOSE TO D-GLUCURONIC ACID, L-GULONIC ACID AND L-ASCORBIC ACID IN NORMAL AND BARBITAL-TREATED RATS

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SUMMARY

1. *In vivo* experiments in rats showed that D-[1-^{14}C]galactose was better than D-[1-^{14}C]glucose as a precursor of D-glucuronic acid, L-gulonic acid and L-ascorbic acid. The conversion of D-[1-^{14}C]galactose to D-glucuronic acid and L-gulonic acid was demonstrated in rat-liver homogenate. The results presented are in accord with the biosynthesis of L-ascorbic acid via the uridine nucleotides.

2. The administration of barbital to rats stimulated the *in vivo* conversion of D-[1-^{14}C]galactose to D-glucuronic acid, L-gulonic acid and L-ascorbic acid. Enhanced formation of D-glucuronic acid from D-[1-^{14}C]galactose was also observed in liver homogenates from rats pretreated with Chloretone.

3. Formation of D-galacturonic acid from D-galactose *in vivo* was not detected in rats.

INTRODUCTION

L-Ascorbic acid is synthesized in rats from D-glucose through the intermediate formation of D-glucuronic acid and L-gulonic acid[1–4]. Since no information has been presented on the role of D-galactose as a precursor of L-ascorbic acid in rats, the possibility that this hexose may also enter this biosynthetic pathway was investigated. The results presented here show that D-[1-^{14}C]galactose is considerably better than D-[1-^{14}C]glucose as a precursor of L-ascorbic acid. Further observations are given which point out a striking effect of drugs in stimulating the metabolism of D-galactose to D-glucuronic acid, L-gulonic acid and L-ascorbic acid.

MATERIALS AND METHODS

Radioactive compounds: D-[1-^{14}C]galactose (2.54 μC/mg) was obtained from the National Bureau of Standards, Washington, D.C.

Abbreviations: ATP, adenosine triphosphate; UDPG, uridine diphosphoglucose; Tris, tris-(hydroxymethyl)aminomethane; DPN, diphosphopyridine nucleotide; and TPN, triphosphopyridine nucleotide.

Experimental animals: Male Wistar rats (272–356 g) were maintained on a basal diet of evaporated milk for at least 10 days prior to each experiment. Metabolism cages were used for the collection of urine.

Isolation procedures: L-Ascorbic acid was isolated as its 2,4-dinitrophenylosazone from the tissues of normal animals and from the urine of barbital-treated animals[3]. The D-glucuronic acid and L-gulonic acid were isolated by specific carrier dilution procedures[3, 5].

The ^{14}C present in urine as D-galacturonic acid was determined as follows: 300 mg of D-galacturonic acid was added to an aliquot of urine and the solution was placed on an Amberlite IR-4B anion exchange column[3]. The D-galacturonic acid was eluted with 2 N formic acid and the eluate evaporated to a syrup. Crystallization of D-galacturonic acid was initiated by the addition of methanol to the syrup. The D-galacturonic acid was converted to mucic acid by oxidation with nitric acid. The mucic acid was washed successively with 5 ml portions of boiling water and plated on glass fiber filter paper. This isolation procedure afforded complete separation of D-galacturonic acid from labeled D-glucuronic acid and L-gulonic acid present in urine.

Degradation procedures: The degradation of carbon-6 of L-ascorbic acid was accomplished by periodic acid cleavage of the primary alcohol group to give formaldehyde which was recovered as its dimedon derivative[6].

In vitro studies: A two stage incubation procedure was used for studying the conversion of D-[1-^{14}C]galactose to D-glucuronic acid in liver homogenates. The conditions were as follows: 3.0 ml of a 30 % liver homogenate in 1.15 % potassium chloride were added to a solution containing 10 mg ATP and 3 mg UDPG in 1.0 ml of 0.25 M phosphate buffer (pH 7.8), 0.1 ml of 0.25 M $MgCl_2$, and 0.2 ml of 1.0 M Tris buffer (pH 8.62). 2 mg of D-[1-^{14}C]galactose in 0.7 ml of water was then added to give a final volume of 5.0 ml and a pH of 7.8. The mixture was incubated in 30-ml beakers with shaking at 37° for 30 min. After cooling in ice, 10 mg of DPN in 1.33 ml of 1 M Tris buffer (pH 8.62), 0.67 ml of 0.6 M nicotinamide, and 3 ml of fresh 30 % liver homogenate were added to give a final volume of 10 ml and a pH of 8.6. The mixture was again incubated for 30 min and cooled. Following the addition of carrier D-glucuronic acid (300 mg), enough trichloroacetic acid solution was added to obtain a final concentration of 5 %. The resulting mixture was centrifuged and the D-glucuronic acid was isolated[3].

Measurement of radioactivity: The methods for the preparation of samples for the assay of ^{14}C have been described[3].

RESULTS

D-[1-^{14}C]Galactose was administered to rats and the amount of ^{14}C present in the body L-ascorbic acid was measured at 24 h. The data in Table I show that the conversion of D-[1-^{14}C]galactose to L-ascorbic acid was about ten-fold greater than that obtained with D-[1-^{14}C]glucose.

92 % of the total ^{14}C of the L-ascorbic acid was in carbon-6 when D-galactose was administered compared to about 60 % found in carbon-6 of the vitamin after the administration of D-[1-^{14}C]glucose.

The conversion of D-[1-^{14}C]galactose to urinary D-glucuronic acid, L-gulonic acid and L-ascorbic acid was also measured in barbital-treated rats (Table II). These

TABLE I

CONVERSION OF D-[1-^{14}C]GALACTOSE AND D-[1-^{14}C]GLUCOSE TO L-ASCORBIC ACID IN RATS

Expt.	*Compound*	*% of dose per mg tissue L-ascorbic acid**	*% of total L-[^{14}C]ascorbic acid in C-6*
1	D-[1-^{14}C]galactose	0.011	86
2	D-[1-^{14}C]galactose	0.015	98
**	D-[1-^{14}C]glucose	0.0015	60

* Labeled L-ascorbic acid was determined by a technique used previously based on the unusually long half-life of L-ascorbic acid[3].

** Average of data obtained previously[3, 11].

TABLE II

CONVERSION OF D-[1-^{14}C]GALACTOSE TO URINARY D-GLUCURONIC ACID, L-GULONIC ACID AND L-ASCORBIC ACID IN RATS

Expt.	*Precursor*	*% of administered ^{14}C in*		
		D-glucuronic acid	*L-gulonic acid*	*L-ascorbic acid*
		Barbital-treated rats		
1	D-[1-^{14}C]galactose	0.60		0.51
2	D-[1-^{14}C]galactose	1.27		0.60
3	D-[1-^{14}C]galactose		0.68	1.00
4	D-[1-^{14}C]galactose	2.09	0.61	0.95
5	D-[1-^{14}C]galactose	2.00		
*	D-[1-^{14}C]glucose	0.41	0.19	0.19
		Normal rats		
6	D-[1-^{14}C]galactose	< 0.08	< 0.05	< 0.04
7	D-[1-^{14}C]galactose	< 0.05	< 0.09	< 0.05
8	D-[1-^{14}C]galactose			< 0.05
*	D-[1-^{14}C]glucose	< 0.03	< 0.03	0.02

* Average of previously published data[5, 11, 12].

conversion values are considerably greater than those obtained previously under the same conditions with D-[1-^{14}C]glucose. The L-ascorbic acid isolated after administration of D-[1-^{14}C]galactose had an average of 83 % of its total ^{14}C in carbon-6 compared to an average value of 60 % obtained in similar experiments with D-[1-^{14}C]glucose.

The results in Table II show striking differences in the conversion of D-[1-^{14}C]-galactose to urinary D-glucuronic acid, L-gulonic acid and L-ascorbic acid in normal and barbital-treated rats. In normal rats no detectable conversion of D-[1-^{14}C]-galactose to these three urinary products was observed while considerable conversion was found to occur in barbital-treated rats. Marked differences were also observed in the conversion of D-[1-^{14}C]glucose to urinary D-glucuronic acid, L-gulonic acid and L-ascorbic acid in normal and barbital-treated rats[5, 11, 12].

In view of the extensive conversion of D-[1-^{14}C]galactose to urinary D-glucuronic acid in barbital-treated rats, the possibility that D-galacturonic acid might be formed from D-galactose was investigated. In the experiments given in Table II, it was found that less than 0.05 % of the administered D-[1-^{14}C]galactose was recovered as urinary D-galacturonic acid in barbital-treated rats. In contrast, the average conversion of D-[1-^{14}C]galactose to D-glucuronic acid under these conditions was 1.5 %.

The conversion of D-[1-^{14}C]galactose to D-glucuronic acid was catalyzed by enzymes in rat liver (Table III). In these experiments the system was fortified with ATP, UDPG and DPN, cofactors which are necessary for the formation of UDP glucuronic acid[7–10]. The conversion of D-[1-^{14}C]galactose to labeled D-glucuronic acid was 3 to 4 times greater in livers from animals pretreated with Chloretone than in livers from non-drug treated animals. Examination of the incubation mixtures showed no detectable formation of labeled L-gulonic acid. Since the reduction of D-glucuronic acid to L-gulonic acid has been shown to be catalyzed by a reduced TPN requiring enzyme[4], the second incubation system was fortified in an additional experiment with 1.0 mg of reduced TPN. The conversion of D-[1-^{14}C]galactose to labeled D-glucuronic acid and L-gulonic acid was 0.46 % and 0.17 %, respectively.

DISCUSSION

The results of this investigation along with evidence previously presented indicate that the most likely pathway for the conversion of D-glucose or D-galactose to L-ascorbic acid is as given in Fig. 1. The enzymes required for the conversion of D-glucose or D-galactose to UDP-glucuronic acid as well as those which take D-glucuronic acid to L-ascorbic acid have been described[4,7–10,13–16]. The formation of D-glucuronic acid from UDP glucuronic acid through D-glucuronic acid-1-PO$_4$ by enzymes in kidney[17] and liver [18] has recently been reported. In this study the overall conversion of D-galactose to D-glucuronic acid and L-gulonic acid in rat liver was demonstrated. Evidence was also presented here that D-galactose is a more direct precursor of

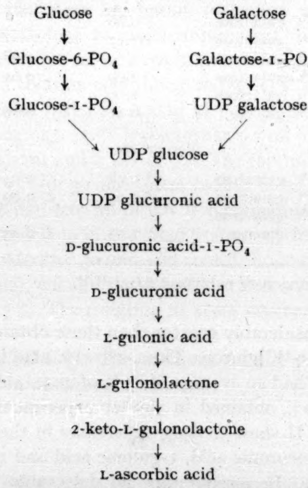
Glucose
Galactose
Glucose-6-PO$_4$
Galactose-1-PO$_4$
Glucose-1-PO$_4$
UDP galactose
UDP glucose
UDP glucuronic acid
D-glucuronic acid-1-PO$_4$
D-glucuronic acid
L-gulonic acid
L-gulonolactone
2-keto-L-gulonolactone
L-ascorbic acid

Fig. 1.

L-ascorbic acid *in vivo* than is D-glucose. This was shown both by the increased conversion of D-[1-^{14}C]galactose to L-ascorbic acid and by finding that its carbon chain underwent considerably less randomization in the formation of the vitamin (Table I). In view of the recent evidence that D-galactose is a more direct precursor of UDPG than is D-glucose[19], the results of the *in vivo* experiments given here are in accord with biosynthesis of L-ascorbic acid via the uridine nucleotides (Fig. 1).

Previous studies with D-[1-^{14}C]glucose have shown that many drugs including barbital, Chloretone, aminopyrine and 3-methylcholanthrene stimulate the metabolism of D-glucose to D-glucuronic acid, L-gulonic acid and L-ascorbic acid[12, 20]. The results of this study show that one of these drugs, barbital, also markedly stimulates the conversion of D-[1-^{14}C]galactose to urinary D-glucuronic acid, L-gulonic acid and L-ascorbic acid. The values for the amounts of D-[1-^{14}C]galactose incorporated into these three metabolites in barbital-treated rats represent minimum values since these compounds are all extensively metabolized in the rat[2, 3, 20]. A drug effect on D-galactose metabolism was also demonstrated *in vitro*. For instance, liver homogenates from rats pretreated with Chloretone converted about 4 times more D-galactose to D-glucuronic acid than did liver homogenates from normal rats (Table III).

TABLE III

CONVERSION OF D-[1-^{14}C]GALACTOSE TO D-GLUCURONIC ACID IN RAT-LIVER HOMOGENATE

Expt.	*Treatment**	*% conversion*
1	None	0.21
2	None	0.18
3	Chloretone	0.88
4	Chloretone	0.68
5	Chloretone	0.77

* Wistar rats (275–325 g) were maintained on a milk diet and were given 40 mg of Chloretone in milk daily for 7 days prior to killing the animals for enzyme assays. The untreated group received only milk. Each experiment represents pooled livers from 3 rats.

The mechanism by which barbital and Chloretone stimulate the synthesis of L-ascorbic acid is not known. This stimulatory effect has been observed with many drugs of unrelated chemical and pharmacological properties[21, 22]. The available evidence indicates that these drugs increase L-ascorbic acid biosynthesis by stimulating the formation of D-glucuronic acid[3, 12, 22]. Glucuronide formation is not required since barbital, one of the most potent drugs in exerting this effect, is recovered unchanged in urine[12]. In addition, borneol, phenophthalein, α-naphthol, and aniline which are potent in forming glucuronides, have no effect on the synthesis of L-ascorbic acid[12, 22]. It is of interest that those drugs which stimulate the synthesis of L-ascorbic acid also increase the activity of liver microsomal enzymes which metabolize various foreign compounds[22]. This stimulatory effect of drugs on L-ascorbic acid biosynthesis and on the liver microsomal enzymes may represent a new adaptive response to foreign compounds.

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FIXATION OF MUSCLE PROTEINS WITH ANTIBODIES

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SUMMARY

Treatment of myofibrils with antibodies, prepared against purified muscle proteins, increases the phase density of well-defined parts of the sarcomere. These parts become insoluble and resist extraction by strong salt solutions. The resulting patterns are different with various antibodies and characteristic to the specific antibody used. Treatment of the myofibrils with antimyosin protects the A band material from extraction. Antibody prepared against light-meromyosin fixes the lateral borders of the A band; antibody prepared against heavy-meromyosin fixes primarily the central region of the A band. Anti-actin alters the extractability of the whole sarcomere.

INTRODUCTION

When antibodies are bound *in situ* by antigens concentrated in an intracellular structure, the phase density of that structure is enhanced[1]. Presumably, this increase in phase density is due to the micro-precipitation of antibody protein on the involved structure. It is also known that formation of antibody-antigen complexes alters the solubility of the reacting antigen. This report demonstrates how these two side effects of intracellular binding of antibodies may be combined and used as a cytochemical technique for the selective fixation and localization of cell constituents under the phase microscope.

Glycerol extracted myofibrils prepared from striated muscle offer several advantages for such a study. Most of the myofibrillar proteins can be accounted for by a few fairly well characterized proteins[2,3] and there is considerable information relating these proteins to the cytological features of the sarcomere[4,5]. Furthermore, rabbit antibodies against muscle antigens of the chicken have been labelled with fluorescein and used to "stain" glycerol-extracted myofibrils. Labelled antibodies have been prepared against myosin, heavy meromyosin, light meromyosin and actin. And while there are still many uncertainties relating to the homogeneity and specificity of both the muscle antigens and antibodies, myofibrils treated with these various antibodies yield patterns which are different and characteristic for each[6–8].

MATERIAL AND METHODS

Myofibrils prepared from the deep pectoral muscles of chickens were kept at rest length in 50 % glycerol at — 20° for two weeks or longer[9]. The muscle was blended in the cold in 25 % glycerol (0.016 *M* pH 7.5 phosphate buffer) and washed several times in the same solution. Aliquots were treated for 1 h against the respective fluorescent antibodies, then washed repeatedly with the glycerol-phosphate solution to remove unbound antibody. In the control experiments the fibrils were treated with fluorescein labelled normal serum. The fluorescein labelled antibodies were the same as used in previous studies[1,7,8]. For extraction, Hasselbach-Schneider solution (H-S) as modified by HANSON AND HUXLEY[10], and 0.6 *M* KI were used. H-S solution is thought to remove myosin selectively from myofibrils, while KI is known to extract actin in addition to myosin[11]. Extraction of myofibrils treated with normal serum or with the different antisera was followed under the phase microscope and allowed to proceed for 10 min. After extraction the fibrils were rinsed with a salt solution of low ionic strength (0.04 *M* KCl, 0.01 *M* pH 7 phosphate buffer).

Though fluorescein labelled normal serum and antisera were used in the following experiments, only those changes that could be observed with the phase microscope are discussed; the changes observed with the fluorescence microscope will be described in another report.

RESULTS

Application of H-S or KI to an untreated fibril or one treated with labelled normal serum, results in conspicuous changes in the phase density of both A and I bands. These changes are due to several events, in addition to removal of protein. Shortly after exposure to the extracting solutions the myofibrils shorten longitudinally and swell laterally; they may shorten to 80 % of rest length and double in width. During this period there is also a shift of the optically dense material that resists extraction. After treatment with H-S solution the Z band appears very prominent and the phase density of the I bands appears greater than the middle of the altered A bands. The center of the former A bands have an exceedingly low density and the width of these areas is wider than the width of H bands of unextracted fibrils. Swelling and shortening of the myofibril is more drastic during KI extraction and the resulting pattern is different. During KI extraction the loss of density in the A bands is followed by disappearance of the Z bands. Material responsible for the remaining A and I band density gradually collects and approaches the position formerly occupied by the Z band. This former A and I band material forms a doublet of dense lines which may fuse into a single band imitating the appearance of a single Z band. These events take about 10 min and result in irregular "sarcomeres" separated by dense doublets or single lines (Fig. 1, 1a,b). Shortening of the fibril and rearrangement of material should be taken into account when extraction studies are employed for the localization of muscle proteins.

The O.D. of the A bands under the phase microscope is enhanced by treating fibrils with antimyosin. When such fibrils are extracted the O.D. of the A bands appears still greater. Whether this latter apparent increase in O.D. is due to extraction of I band material or whether I band material is incorporated into the A band, has not been determined. Following the extraction procedures the dimensions of the A bands remain unaltered, though in some instances there is a partial clearance of the center of the A band. After treatment with H-S solution the Z membranes remain and the total sarcomere pattern, aside from the increased density of the A bands, appears similar to the normal unextracted fibril. KI affects mostly the I band region of a fibril treated with antimyosin. The Z bands disappear and the I bands often shorten so that the A bands may lie closer together than in a normal fiber. In brief, treating a fibril with antimyosin appears to fix A band material so as to withstand the action of extracting solutions (Fig. 2).

Fibrils treated with antibody prepared against light meromyosin exhibit increased phase densities along the lateral borders of the A band; these are the regions that are protected against the action of extracting solutions. Upon addition of H-S solution the center of the A band swells and becomes clear, its density falling below that of the I band. This central clear zone of the A band is broader than an H band and it is bordered by dark narrow bands which correspond to the edges of the erstwhile A band. In KI solutions the dense lateral edges of the A band separate and converge on the position of the Z band which disappeared, forming doublets of dense lines. Each doublet is formed from the displacement of the remnants of two adjoining A bands. Doublets formed in fibrils treated with anti-light meromyosin do not fuse as they do in control extracted fibrils (Fig. 3).

Fibrils treated with antibody against heavy-meromyosin invariably exhibit strong M bands under the phase microscope. When fibrils are treated for a short time (15 min) with anti-heavy meromyosin this may be the only region of the A band resistant to extraction (Fig. 4a). In fibrils stained for 1 h the strong M bands are thicker and flanked by dense A bands after extraction (Fig. 4b). Particularly after KI, the remaining A bands are often narrower than the normal A bands and the I bands appear to be wider than in unextracted fibers. The apparently greater density and thickness of the M bands after extraction might be due to material converging on this area.

Fibrils treated with anti-actin for 1 h exhibit I, A and Z bands; H bands are never present, but M bands are prominent. H-S extraction produces little change in the sarcomere pattern other than making the Z band sharper. But after KI, M bands appear sharper and thicker, again suggesting that material might accumulate in this region during strong extraction (Fig. 5).

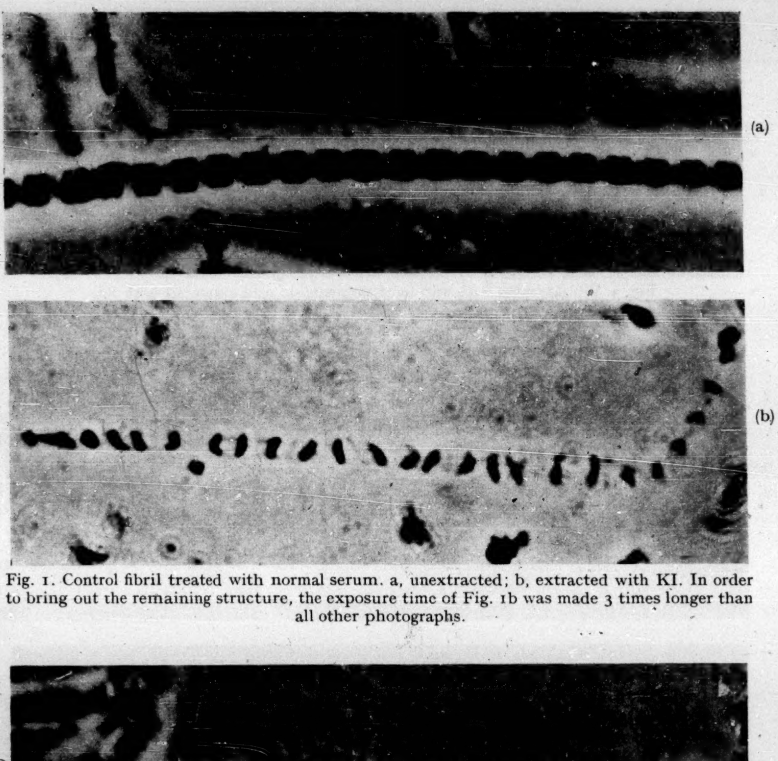

Fig. 1. Control fibril treated with normal serum. a, unextracted; b, extracted with KI. In order to bring out the remaining structure, the exposure time of Fig. 1b was made 3 times longer than all other photographs.

Fig. 2. Fibril treated with anti-myosin and extracted with KI.

CONCLUSIONS

Antibodies prepared against proteins of the myofibril enhance the phase density of circumscribed parts of the sarcomere, and by conferring an insolubility to these parts, result in their resisting extraction by strong salt solutions. The localization of these resistant regions coincides with the localization of the antigens as established by the staining patterns observed under the fluorescent microscope. Thus, while antimyosin acts along the entire length of the A band, the action of antibody prepared against

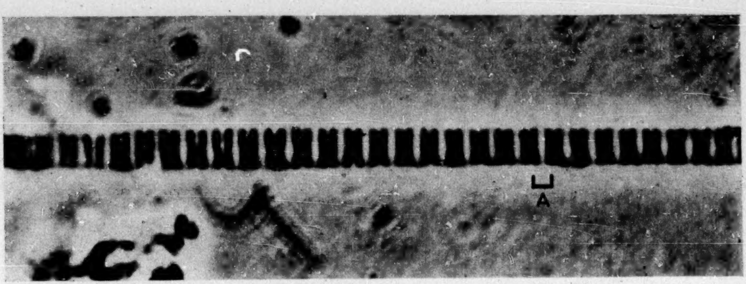

Fig. 3. Fibril treated with anti-light meromyosin and extracted with KI.

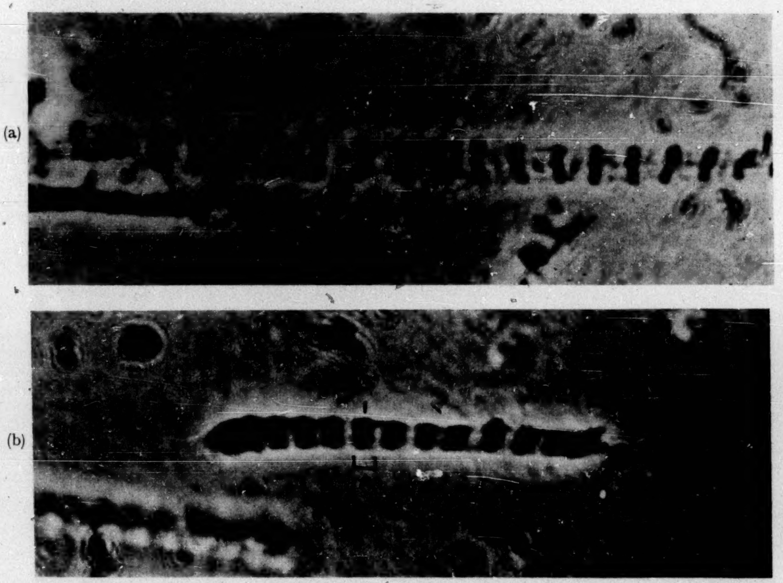

Fig. 4. Fibril treated with anti-heavy meromyosin and extracted with KI. a, 15-min, treatment; b, 60-min treatment.

light-meromyosin is restricted to the lateral borders of the A band, and the effect of antibody prepared against heavy-meromyosin is directed mainly to the central portion of the A band. Though the antibodies confer resistance to extraction, the regions involved may still shift laterally during the extraction procedures. Whether A band material shifts toward the Z band or towards the M band depends upon which antibody is used. Such shifting of contractile proteins during extraction should be considered when correlating the proteins with their normal location in the sarcomere.

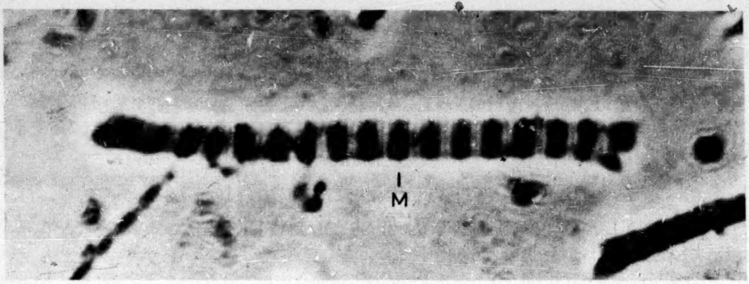

Fig. 5. Fibril treated with anti-actin and extracted with KI.

ACKNOWLEDGEMENTS

This work originated from a discussion with Dr. D. MAZIA, University of California, who suggested that the use of antibodies may make certain muscle proteins resistant to extraction.

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DETERMINATION OF AMINO ACIDS AS PHENYL THIOHYDANTOIN DERIVATIVES

III. QUANTITATIVE DETERMINATION OF 3-PHENYL-2-THIOHYDANTOINS FROM PAPER CHROMATOGRAMS

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SUMMARY

A method for quantitative determination of the 3-phenyl-2-thiohydantoin derivatives (PTH) from the naturally occurring amino acids is described. The PTH's are synthesized by a micro synthetic procedure from protein hydrolysates (0.5–1 mg protein) and separated on paper chromatograms. The spots are located with the aid of a fluorescent screen and eluted from the paper with ethanol. The quantitative calculations are based on the optical density at 269 and 320 mμ of the ethanolic eluates.

INTRODUCTION

The phenyl thiohydantoin (PTH) derivatives from amino acids were first introduced in protein chemistry by P. EDMAN in his method for stepwise degradation of peptides[1,2]. In connection with this work a paper chromatographic procedure was worked out for separation and identification of the PTH's released by this technique[3], a method which later was improved[4].

The use of PTH's for quantitative determination of the natural amino acids in protein hydrolysates was described in 1956[5] and a full description of this procedure was given in two papers[6,7], the first of which dealt with a microsynthetic procedure for the conversion of minute amounts of amino acids into PTH derivatives and the second which gave detailed descriptons of their separation and estimation by means of column chromatography.

The rapid and satisfactory separation and the ease by which these derivatives could be identified on paper chromatograms[4] prompted us to work out a quantitative paper chromatographic procedure for these compounds. In simplicity and accuracy this method seems to be more convenient than similar methods hitherto published for amino acids analysis. It takes advantage of the ease by which PTH amino acids can be separated due to their good solubility in different organic solvents, and to the simplicity in identification and estimation owing to the high and uniform u.v.-absorption of these derivatives.

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EXPERIMENTAL

Conversion of amino acids into PTH derivatives

Though this procedure has already been outlined[6] it is presented here to give a full description of the method.

All operations are performed in tubes of transparent quartz, length 150 mm, inner diameter 8–10 mm. The tubes are cleaned in ethanol, dried and ignited.

Reagents used in this step are:

Phenyl isothiocyanate (Eastman Kodak). Distilled *in vacuo*.

Acetone, *pro analysi* (Merck). Refluxed with potassium permanganate, and afterwards stored in contact with the same agent. The desired quantity is distilled off immediately before use. b.p. 56°.

Triethylamine (Eastman Kodak). Refluxed for 3 h with phthalic acid anhydride (5 g/100 ml), and the desired quantity distilled from a Widmer column immediately before use. b.p. 89°.

Acetic acid, *pro analysi* (Merck). Distilled. b.p. 118°.

Buffer. This is made up from 2.00 ml 2 *N* acetic acid, 1.20 ml triethylamine, and distilled water to 25.0 ml. Mixed with an equal volume of acetone, a pH of 10.1 results.

Hydrogen chloride saturated acetic acid. Hydrogen chloride is generated by pouring sulfuric acid into concentrated hydrochloric acid and the gas is washed by passing through concentrated sulfuric acid. The acetic acid is thoroughly saturated in a quartz tube with hydrogen chloride at room temperature.

Amino acids used are those specified in a preceding paper[6].

For the synthesis of the PTH's approx. 5 μmoles amino acids are dissolved in 250 μl buffer in a quartz tube. 250 μl of a solution containing 6 μl phenyl isothiocyanate/ml acetone is added, and the tube is gently shaken to ensure complete mixing, stoppered, and placed in a water bath at 25° for 2.5 h. It is then placed in a desiccator over phosphorous pentoxide and the content is carefully evaporated for 15 min using a water pump and finally over night with an oil pump giving a vacuum of approx. 0.001 mm Hg (cold trap).

In order to transform the phenylthiocarbamyl (PTC) derivatives to PTH amino acids the dry residue is subsequently dissolved in 100 μl glass distilled water and 200 μl acetic acid saturated with hydrogen chloride is added. The tube is gently shaken, stoppered, and left in a water bath at 25° for 6 h. Afterwards, the solvent is evaporated in a desiccator over potassium hydroxide in the way already described. A blank without amino acids is treated in the same way as above.

In the case of amino acid analyses of proteins or peptides, 0.5–1 mg of the sample is transferred to a quartz tube, and 0.3 ml twice glass distilled hydrochloric acid (5.7 *N*) is added. The tube is filled with nitrogen gas, sealed, and kept at 110° for 22 h. After cooling the tube is opened and the content evaporated to dryness in a desiccator *in vacuo* over KOH. To remove remaining hydrochloric acid the hydrolysate is repeatedly evaporated after adding water. For conversion of the amino acids in the hydrolysate into PTH's, 250 μl buffer, and 250 μl acetone–phenyl isothiocyanate solution is added to the dry residue, and subsequently the descriptions given above are strictly followed.

As cysteine or cystine do not form PTH derivatives these amino acids have to be oxidized to cysteic acid before coupling with phenyl isothiocyanate. The oxidation is performed with a slight modification of the performic acid oxidation method given by HIRS[7]. 1.0 ml 30 % H_2O_2 and 9.0 ml 99 % formic acid are mixed and left for 2 h at 25°. About 1 mg protein is dissolved in 0.2 ml 99 % formic acid and 0.2 ml performic acid is added. After mixing, the oxidation takes place at 25° for 2 h and subsequently the solvent is carefully evaporated to dryness in a desiccator *in vacuo* over pellets of KOH. Remaining acid is removed by repeated evaporation after adding water. The oxidized protein is then hydrolyzed with 5.7 *N* HCl and converted to PTH's according to the directions given above.

Paper chromatographic procedure

Throughout the investigation descending chromatography has been performed in glass tanks (20 × 30 cm and 60 cm high). The vessels are closed with glass plates tightened with stopcock grease. Chromatography in four different solvent systems gives an almost complete separation of all PTH's derived from the natural amino acids of a protein hydrolysate.

The reagents used in this step are: 1. *n*-heptane (Eastman Kodak). Distilled, b.p. 98–99°. 2. Ethylene chloride, *puriss* (Fluka). Distilled, b.p. 83°. 3. Formic acid, 98–100 %, *pro analysi* (Merck). Diluted with water to desired concentration. 4. Propionic acid (Eastman Kodak) is refluxed for 3 h with chromic acid (Merck, *pro analysi*, 5 g/150 ml). After cooling the propionic acid is poured off, distilled on a Widmer column, and the fraction boiling at 140–141° is collected.

The composition of the four solvent systems are given in Table I. Solvents I, II, and III give two phases. After mixing and separation the lower one is discarded. Solvent IV gives only one phase. New solvents are mixed for each run, and this is particularly important with solvent IV in which esterification between butanol and formic acid occurs on standing.

TABLE I

VOLUMES OF THE DIFFERENT ORGANIC SOLVENTS IN CHROMATOGRAPHIC SYSTEMS I, II, III, AND IV

	Solvent system			
	I	*II*	*III*	*IV*
Heptane	60	60	30	40
Ethylenchloride	5	30	60	
Propionic acid	5			
n-butanol				30
Formic acid, 75 %	5	5	5	9

The short sides of the chromatographic jars are lined with filter papers soaked in moving phase which covers the bottom of the jars to a height of approx. 1 cm. Fresh moving phase is added to these papers in jar I, II, and III before each run. At the bottom of each jar a dish is placed, containing stationary phase, into which a filter paper (width approx. 15 cm) is hung. The stationary phase in jar I and II consists of 90 % formic acid, in jar III of 75 % formic acid, and in jar IV of 25 % formic acid. Before each run a new portion of stationary phase is sprayed over the hanging paper strip in jars I, II, and III. At the end of each week the moving and stationary phases which equilibrate tanks I, II, and III are replaced. Jar IV is allowed to equilibrate with moving and stationary phase for 2–3 days before running a chromatogram. In this jar a new portion of moving and stationary phase is added only when consumed. The jars are kept at 24°.

Papers used in this method (exclusively Whatman No. 1) are cut out according to the layout in Fig. 1, and are pretreated by soaking in a 0.1 % aqueous solution of EDTA-disodium salt. Afterwards the papers are dried at 100° for 0.5–1 h. Generally each paper contains seven or nine strips. The test solution is applied to the central strip and to the strips to the right and left, sample and blank solutions are added (see Fig. 1).

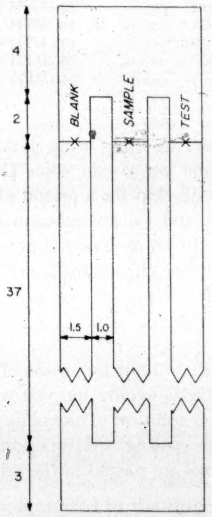

Fig. 1. Drawing of a paper chromatogram with three strips. Application points indicated. Dimensions in cm.

Application is performed using carefully calibrated constriction pipettes (Carlsberg pipettes). It is important that the tip of the pipette rests on the paper throughout the application, thereby insuring constant volumes. When a larger volume than 10 μl is applied, a stream of nitrogen gas is blown from underneath to get the solution rapidly dried.

Four papers, one for each solvent system, are prepared for each amino acid determination and usually 5, 10 and 20 μl of the sample as well as the blank solutions are applied to each paper.

Two test solutions are prepared, designated A and B (see Table II), containing the PTH derivatives from the naturally occurring amino acids. About 5 μmoles of each PTH amino acid are dissolved in 2 ml 90 % acetic acid. For chromatography 10 μl of solution A is applied to chromatograms I and II and 10 μl of solution B to chromatograms III and IV. The test solutions are stored at 5°. At this temperature they are stable for several months.

TABLE II

THE COMPOSITION OF TEST SOLUTIONS A AND B

The figures refer to the spots on chromatograms in Fig. 2.

A	*B*
1. PTH-leucine	2. PTH-*iso*leucine
3. PTH-valine	4. PTH-proline
5. PTH-phenylalanine	9. PTH-glycine
6. PTH-methionine	10. PTH-lysine
7. PTH-alanine	11. Phenylthiourea
8. PTH-tryptophan	12. PTH-tyrosine
9. PTH-glycine	14. PTH-glutamic acid
12. PTH-tyrosine	15. PTH-aspartic acid
13. PTH-threonine	17. PTH-arginine
16. PTH-serine	18. PTH-histidine
	19. PTH-cysteic acid

After application the papers are hung in the jars, and allowed to equilibrate for 15 min in jars I, II, and III, and for 30 min in jar IV. Moving phase is then added, and the papers are allowed to develop for a period of 2.5–3.5 h and attain a length of about 35 cm. Papers I, II, and IV are subsequently dried at 100° for exactly 30 min and paper III for exactly 1.5 h. Exact time and temperature for drying the papers are important especially for chromatogram III from which PTH serine and PTH threonine are determined.

Quantitative evaluation

The spots are located with u.v. light by means of a fluorescent screen[4]. We now use a sheet of transparent plexiglas evenly sprayed with a suspension of fluorescent zinc silicate in chloroform. A hot solution of plexiglas chips in isoamylacetate is then sprayed on to give a protective coating. Corresponding to the cut out strips of the chromatograms, black tape strips are pasted to the screen. This highly facilitates the locating of the spots.

The spots are marked and cut out with a margin of approx. 3 mm. Paper ribbons corresponding in size and level of the spots are also cut out from the blank strip.

The cut out pieces are eluted with 2 ml 95 % ethanol for 1 h at 40° in glass stoppered test tubes. The extinction of the ethanolic eluates is read at 269 mμ in a Beckman DU (light path 1 cm). In addition the samples containing PTH serine and PTH threonine are read at 320 mμ. For calculations of the amount of each amino acid the blank values are subtracted from the sample values. If the time does not allow to cut out, elute and measure the samples the same day, the chromatograms are kept in the cold over night. This does not interfere with the result.

In Table III a factor "C" for the different amino acids is given. This factor put into the equation given below simplifies the calculations of the amino acid content of a sample. "C" represents the recovery of each amino acid under the standard conditions.

$$A = \frac{C \times \varepsilon \times a}{b \times w}$$

where A is amount amino acid of the sample in μmoles/mg; C, coefficient for each individual amino acid, determined by dividing the amount of an amino acid applied

TABLE III

EXTINCTION VALUES AT 269 OR 320 mμ OF THE ELUTED PTH's

Each figure represents the mean value of ten analyses beginning with the micro synthetic procedure for each sample of a synthetic amino acid mixture.

Amino acid	*0.01 μmoles*	*0.02 μmoles*	*0.04 μmoles*		*Yield in %*	*C*
			Found	*Calculated*		
Alanine	0.084	0.167	0.334	0.320	104.2	0.120
(Ammonium chloride)*		0.060	0.121			0.330
Arginine	0.070	0.143	0.284	0.318	89.4	0.141
Aspartic acid	0.076	0.154	0.306	0.322	95.0	0.131
Cysteic acid	0.077	0.158	0.305	0.332	92.0	0.131
Glutamic acid	0.075	0.153	0.304	0.318	95.6	0.132
Glycine	0.071	0.147	0.290	0.298	97.4	0.138
Histidine	0.061	0.124	0.250	0.310	80.6	0.160
Hydroxyproline	0.066	0.127	0.254	0.272	93.4	0.157
Isoleucine Leucine	0.081	0.157	0.308	0.337	91.5	0.130
Lysine**	0.094	0.222	0.455	0.580	78.5	0.088
Methionine	0.086	0.172	0.345	0.342	100.8	0.116
Phenylalanine	0.075	0.151	0.294	0.310	94.8	0.136
Proline	0.071	0.136	0.274	0.286	95.8	0.146
Serine***	0.058	0.113	0.221			0.181
Threonine***	0.082	0.157	0.300			0.133
Tryptophan	0.091	0.176	0.340	0.394	86.8	0.118
Tyrosine	0.079	0.148	0.297	0.312	95.2	0.135
Valine	0.073	0.148	0.300	0.330	90.9	0.133

* Determined as phenylthiourea.

** Determined from chromatogram II.

*** Measured at 320 mμ.

to the paper by the extinction (Example, 0.04 μmoles glycine applied, ε at 269 mμ = 0.290, $C = 0.04/0.290 = 0.138$); ε, extinction value read at 269 mμ (or 320 mμ); a, μl 90% acetic acid in which the sample is dissolved before application to the chromatogram; b, μl of solution a applied to the chromatogram; w. weight of sample in mg.

RESULTS AND DISCUSSION

From the chromatograms in Fig. 2 it can be seen that nearly all PTH derivatives from amino acids occurring in proteins can be separated using four one-dimensional chromatograms. The exceptions are PTH leucine and PTH isoleucine, PTH proline, PTH lysine and PTH serine. As to the quantitative determination of these derivatives a special comment is necessary. PTH leucine and PTH isoleucine travel as one spot in chromatographic system I as well as II. Consequently the amount of these PTH's are determined together. On chromatogram I PTH proline travels together with PTH phenylalanine whereas on chromatogram II PTH proline moves with PTH valine. As PTH valine and PTH phenylalanine are determined, without interference, from chromatogram I and II respectively, PTH proline is estimated by subtracting their respective extinction values either from the sum of PTH proline plus PTH valine (II) or from the sum of PTH proline plus PTH phenylalanine (I). Both procedures give the same value for PTH proline.

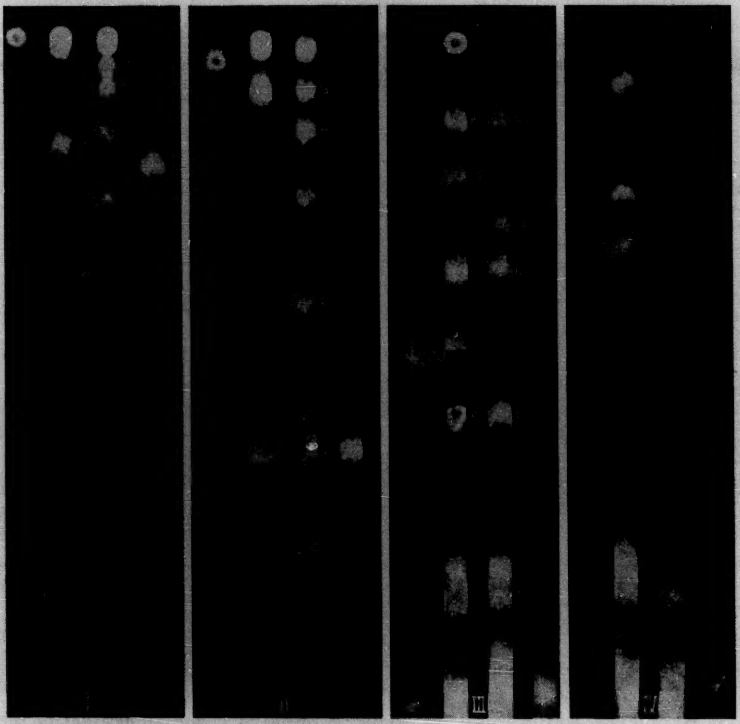

Fig. 2. u.v. photocopies (negatives) of strips run in systems I, II, III, and IV, showing the separation of the PTH's of a synthetic amino acid mixture. The figures to the right of the spots refer to the derivatives given in Table II. In addition, numbers 20 and 21 represent PTH hydroxyproline and diphenylthiourea respectively.

The amount of PTH lysine is similarly determined. On chromatogram II this derivative travels together with phenylthiourea (or PTH hydroxyproline; see later) and PTH glycine, and on chromatogram III together with PTH alanine and PTH tryptophan. Phenylthiourea and PTH glycine on chromatogram III, and PTH alanine and PTH tryptophan on chromatogram II move as single spots. Consequently, the amount of PTH lysine can be determined by subtraction, if the extinction values of the other derivatives are known. Normally, PTH lysine is best calculated from chromatogram II.

As tryptophan is destroyed during the hydrolysis in hydrochloric acid, the amount of this amino acid in proteins or peptides has to be estimated by one of the conventional methods described.

On chromatogram III, PTH serine moves with PTH aspartic acid. This behaviour, however, does not influence the quantitative determination of this derivative. PTH serine and PTH threonine, as distinguished from all of the other PTH amino acids, undergo decomposition through dehydration resulting in an extra absorption peak at 320 mμ[8]. In the present method this reaction occurs when drying the paper chromatograms. The influence on the decomposition at 100° of PTH serine and PTH threonine when drying a paper chromatogram at different intervals is shown in Fig. 3. As can be seen PTH serine reaches its highest extinction value at 320 mμ after a drying period for 1.5 h, and simultaneously the extinction at 269 mμ decreases to nearly its lowest value. The standard condition for this method is therefore to dry chromatogram III for exactly 1.5 h at 100° while the chromatograms I, II, and IV are dried for 30 min. This prescription allows us to determine PTH serine and PTH threonine by measuring the extinction of their eluates at 320 mμ. Under these circumstances the ratio $\varepsilon_{269}/\varepsilon_{320}$ for PTH serine is constant 0.43. This quotient should be multiplied by the extinction value for PTH serine at 320 mμ. The product thus obtained is subtracted from the sum of PTH aspartic acid and PTH serine at 269 mμ; the difference is the absorption for PTH aspartic acid at this wave length. As the spot of PTH threonine on chromatogram III does not interfere with other spots, this derivative is estimated solely by measuring the alcoholic extract at 320 mμ.

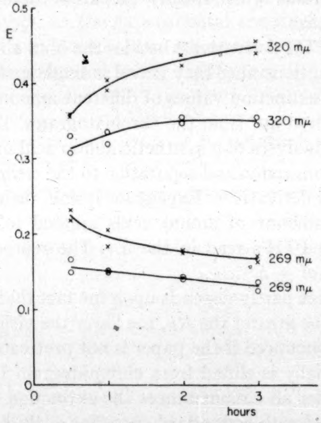

Fig. 3. Influence of varying drying times of chromatogram III at 100° on the extinction values of PTH serine (○) and PTH threonine (×) eluates.

Ammonium ions occurring in a specimen to be analyzed also react with phenyl isothiocyanate to give phenyl thiourea. As this compound has about the same absorption spectrum in u.v. as the PTH amino acids, the amount of ammonium can be determined by analyzing the phenyl thiourea spot on chromatogram III at 269 mμ. The result of such an analysis with different amounts of ammonium chloride is given in Table III. If the sample does not contain ammonium salts, the amount of phenylthiourea in the analysis reflects the content of amide groups split off during acid hydrolysis. However, PTH hydroxyproline interferes with this estimation, as it possesses the same R_F as phenylthiourea. Generally, this interference causes no difficulty with ammonium determinations, as hydroxyproline is not included in most proteins or peptides.

As cysteine and cystine do not form PTH derivatives, these amino acids first have to be oxidized to cysteic acid[7] before coupling with phenyl isothiocyanate. The extinction values at 269 mμ of PTH cysteic acid eluted from chromatogram IV are given in Table III. It is worth mentioning that PTH cysteic acid derived from a performic acid oxidized protein sometimes has higher R_F-value on chromatogram IV than the corresponding test spot and also travels as a sharp streak. This behaviour is presumably a "salt effect" since authentic PTH cysteic acid applied to such a sample disappears from its right place on the chromatogram, while a proportional increase in the extinction value occurs in the eluted streak.

On the blank strips sometimes a slight spot appears between PTH phenylalanine and PTH valine on chromatogram I, and on the place for PTH valine and PTH proline on chromatogram II. It has the same R_F value as diphenyl thiourea, the reason why this compound has been applied to the chromatograms in Fig. 2. This spot cannot be seen on the chromatograms when freshly prepared triethylamine is used for the buffer in the coupling stage.

Estimation of the PTH derivatives, besides the ones already discussed, does not require any special precautions since they travel as single spots on the chromatograms.

Table III gives the extinction values of different amounts of PTH's eluted with 2 ml 95 % ethanol for 1 h at 40° from the chromatograms. The figures give the mean values for ten different analyses of a synthetic amino acid mixture and represent the over all yield from the conversion and separation to the estimation of the amino acids as phenyl thiohydantoin derivatives. Except for lysine, their exists a linear proportionality between the amount of amino acids applied to the procedure and the extinction values of the PTH's read in the u.v. The reproducibility in each determination is of the order of ± 4%.

The low yield of lysine partly depends upon the fact that its PTH derivative tails on the chromatogram. The greater the R_F, the lower the yield which is obtained. This phenomenon is more pronounced if the paper is not pretreated with EDTA. For that reason PTH lysine normally is eluted from chromatogram II where it has a low R_F value. Nevertheless, under all circumstances the extinction value obtained for PTH lysine has to be compared with a standard curve for evaluation of the lysine content (Fig. 4).

The yield of histidine, which also is low by the present method, can be increased by eluting the PTH histidine from the paper with 70 % ethanol instead of 95 %. This

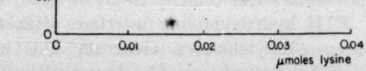

Fig. 4. The relation between the lysine content and the extinction value at 269 mμ of the PTH lysine eluted from chromatogram II.

is also true for the yields of PTH cysteic acid and PTH arginine. However, as the yield of PTH histidine is very reproducible with 95 % ethanol elution we use one procedure to elute all the PTH's from the chromatograms in order to standardize the method as much as possible.

In Table IV, a comparison has been made between our results of the amino acid composition of bovine serum albumin (Armour's) and the values given by STEIN AND MOORE[9]. All of our samples were hydrolyzed with 5.7 *N* HCl at 110° for 22 h. It is known that serine and threonine decompose to a considerable degree during this procedure[10,11]. HIRS, STEIN AND MOORE[11] by hydrolyzing a comparable amino acid mixture for varying times and then extrapolating to zero time, estimated the loss of serine to be 16 % and of threonine to be 8 % after 20 h hydrolysis in 6 *N* HCl at 110°. Our figures for serine and threonine in Table IV have not been corrected for losses during hydrolysis, as one hydrolysis time was used in all determinations, making such an extrapolation impossible. However, it would seem reasonable to expect similar losses during our hydrolyses, as the experimental conditions are virtually identical with those of HIRS, STEIN AND MOORE. If we adjust our results for serine and threonine by adding their values for estimated losses, satisfactory agreement obtains.

TABLE IV

THE AMINO ACID COMPOSITION OF BOVINE SERUM ALBUMIN

Amino acids	*µmoles amino acid/mg protein*		
	This work		STEIN AND MOORE[9]
	1	*2*	3
Alanine	0.734	0.680	0.701
Arginine	0.309	0.301	0.339
Aspartic acid	0.730	0.784	0.820
Glutamic acid	1.069	1.130	1.122
Glycine	0.262	0.269	0.242
Histidine	0.246	0.258	0.258
Isoleucine Leucine	1.109*	1.136*	1.134
Lysine	0.835	0.900	0.877
Methionine	0.066	0.060	0.054
Phenylalanine	0.396	0.389	0.399
Proline	0.406	0.402	0.413
Serine	0.340**	0.336**	0.402***
Threonine	0.441**	0.430**	0.490***
Tyrosine	0.326	0.312	0.279
Valine	0.546	0.530	0.505

* Calculated as leucine.
** Uncorrected for losses during the acid hydrolysis.
*** Corrected for losses during the acid hydrolysis.

As to the results of the other amino acids in Table IV, good correlation exists between the results obtained with the two methods.

The method here described has been extensively used in our laboratory in the past year. When used routinely for analyzing peptides, in conjunction with EDMAN's degradation technique[2], it has proved a reliable and astonishingly simple method.

ACKNOWLEDGEMENTS

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HYDROLYSIS OF NUCLEOSIDE DI- AND TRIPHOSPHATES BY CRYSTALLINE PREPARATIONS OF YEAST INORGANIC PYROPHOSPHATASE

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SUMMARY

1. Crystalline preparations of yeast inorganic pyrophosphatase catalyze phosphate liberation from ATP and ADP, as well as from pyrophosphate, in the presence of zinc ions.

2. A variety of other nucleoside di- and triphosphates are hydrolyzed in the presence of zinc ions and pyrophosphatase, and manganous and cobaltous ions can partially substitute for zinc in effecting ATP hydrolysis.

3. Although several lines of evidence suggest that a single protein possesses Mg^{++}-pyrophosphatase and Zn^{++}-ATPase activities, the possibility cannot be eliminated that a different protein, present in small amounts, accounts for the ATPase activity.

4. Evidence has been obtained that the hydrolysis of ATP occurs primarily between the β- and γ-phosphate groups to yield ADP, which is then converted to AMP.

Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; DPN, diphosphopyridine nucleotide.

[*] The experimental data in this paper are taken from a thesis submitted by M. J. SCHLESINGER in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Chemistry in the Rackham School of Graduate Studies of The University of Michigan.

INTRODUCTION

Yeast inorganic pyrophosphatase is generally believed to act on no other substrate than inorganic pyrophosphate. BAILEY AND WEBB[1] reached this conclusion in working with an extensively purified preparation of the enzyme and reported that the enzyme specifically required magnesium ions. In 1952 KUNITZ[2] described the isolation of crystalline pyrophosphatase from bakers' yeast and found that in the presence of Mg^{++} ions the enzyme did not catalyze the hydrolysis of such compounds as ADP, ATP, or thiamine pyrophosphate. He also observed that Co^{++} or Mn^{++} ions could substitute for Mg^{++} in pyrophosphate hydrolysis but that they were less effective in promoting catalytic activity.

In the course of studies in this laboratory on the ATP-dependent carboxylation of β-methylcrotonyl CoA[3] and on ATP degradation by pyruvic kinase in the presence of carbon dioxide, hydroxylamine, and zinc ions[4], preliminary evidence was obtained[5] that yeast inorganic pyrophosphatase may have wider specificity than generally recognized. The experiments outlined below indicate that crystalline preparations of inorganic pyrophosphatase attack certain organic pyrophosphate esters under conditions not previously tested, *i.e.*, with certain metals other than magnesium. Our findings suggest that pyrophosphatase may have a broader metabolic role than previously thought, and that considerable caution must be used in interpreting the effect of pyrophosphatase when added to crude enzyme systems containing divalent metal ions other than magnesium.

METHODS AND MATERIALS

Lyophilized, crystalline preparations of yeast inorganic pyrophosphatase, kindly provided by Dr. M. KUNITZ, were dissolved in deionized water and stored at 4°. A partially purified preparation of the enzyme, free of ATPase, was made from yeast according to the directions of HEPPEL AND HILMOE[6]. Protein concentrations were determined spectrophotometrically by the method of WARBURG AND CHRISTIAN[7]. The nucleoside di- and triphosphates, AMP, and DPN were commercial preparations.

In a typical assay for ATPase activity, 2 μmoles ATP were incubated with 4 μmoles zinc acetate, 100 μmoles Tris–maleate buffer, pH 6.0, and 10 μg crystalline pyrophosphatase in a final volume of 3.0 ml. The enzyme was omitted from a control tube, and the amount of phosphate found was subtracted from that in the complete tube. After incubation for 30 min at 38° (or 15 min when pyrophosphate served as substrate) the mixtures were deproteinized with trichloroacetic acid (10 % final concentration), and aliquots were taken for phosphate determination[8]. Cysteine was occasionally added to the usual reagents[9] but with no apparent effect on color development. AMP was estimated according to the method of KALCKAR[10].

RESULTS

The liberation of phosphate from ATP in the assay system described above is dependent upon the presence of pyrophosphatase as well as zinc ions (Table I). Other experiments have shown that zinc chloride can replace zinc acetate and that the boiled enzyme is inactive. A lower, but significant, activity is obtained with cobaltous and manganous ions, whereas magnesium and other metals tested are apparently without effect (Table II). The metal concentrations recorded are those at which the highest activity was found in the range $1.6 \cdot 10^{-3}$ to $1.0 \cdot 10^{-2}$ *M*. Various nucleoside

TABLE I

DEPENDENCY OF ATP HYDROLYSIS ON COMPONENTS OF ASSAY SYSTEM

Reaction mixture	*Phosphate formed (µmoles)*
Complete	1.80
No Zn^{++}	0.12
No enzyme	0.12
No ATP	0.00

TABLE II

METAL REQUIREMENT FOR ATP HYDROLYSIS

The reaction mixtures contained 5 µmoles ATP, 50 µg crystalline pyrophosphatase, and the following salts at the concentrations indicated: zinc acetate, cobalt chloride, manganese chloride, magnesium chloride, ferrous sulfate, ferric chloride, potassium aluminum sulfate, cupric sulfate, calcium chloride, and nickel acetate.

Metal added	*Molarity × 10^3*	*Phosphate formed (µmoles)*
Zn^{++}	3.3	4.20
Co^{++}	6.6	0.86
Mn^{++}	6.6	0.94
Ni^{++}	6.6	0.10
Mg^{++}	1.6	0.04
Fe^{++}	6.6	0.10
Fe^{+++}	3.3	0.01
Cu^{++}	3.3	0.06
Al^{+++}	6.6	0.01
Ca^{++}	3.3	0.00

TABLE III

HYDROLYSIS OF VARIOUS NUCLEOSIDE PHOSPHATES

50 µg of pyrophosphatase were employed with 4 µmoles of the triphosphates and 1 µmole of the diphosphates.

Substrate tested	*Phosphate formed (µmoles)*
Adenosine triphosphate	5.40
Guanosine triphosphate	3.62
Uridine triphosphate	3.58
Cytidine triphosphate	4.75
Inosine triphosphate	3.75
Adenosine diphosphate	0.72
Guanosine diphosphate	0.60
Uridine diphosphate	0.62
Cytidine diphosphate	0.46
Inosine diphosphate	0.38
Adenosine 5′-phosphate	0.00

di- and triphosphates were examined as possible substrates in the presence of zinc ions, and the crystalline pyrophosphatase preparation was found to have broad specificity, as shown in Table III. These compounds were not attacked, however, when Mg^{++} was substituted for Zn^{++} or enzyme was omitted. Additional experiments have shown that Co^{++} and Mn^{++} can substitute for Zn^{++} in ADP hydrolysis. DPN is apparently not attacked by pyrophosphatase and Zn^{++}, as judged by the finding that AMP is not liberated.

The pH optimum of the ATPase activity is about 6.0, as shown in Fig. 1, with appreciable activity in the broad range 5.2 to 8.0. KUNITZ[2] obtained pH optima of about 6.3 and 7.2 for Mg^{++}-activated pyrophosphatase, the different values being obtained at different metal–substrate ratios. Experiments at pH 6 with varying concentrations of substrate and metal ions have shown that the optimal Zn^{++}: ATP ratio is 2.0 (Fig. 2). The corresponding values for Zn^{++}: ADP and Zn^{++}: pyrophosphate appear to be about 2.0 and 0.5, respectively. The relation of enzyme activity to substrate concentration is shown in Fig. 3; these and other data on the zinc-dependent reactions are complex and do not justify the calculation of precise K_m values. On the other hand, it is apparent that relatively low ATP and ADP concentrations are adequate to give significant reaction rates.

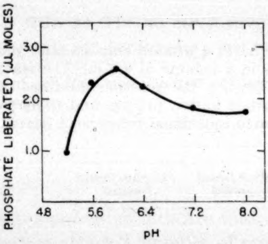

Fig. 1. Liberation of phosphate from ATP as a function of pH. The standard assay was employed with 2 μmoles ATP, 4 μmoles Zn^{++}, 50 μg crystalline enzyme, and 100 μmoles Tris–maleate buffer at the pH values indicated.

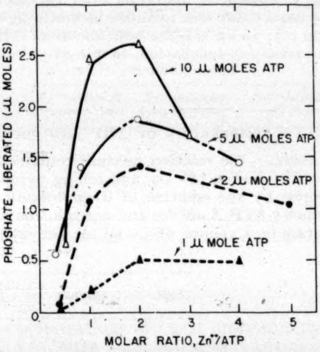

Fig. 2. Phosphate liberation from ATP as a function of the molar ratio of Zn^{++} to ATP. 10 μg pyrophosphatase were employed, with the ATP concentration at the four levels indicated.

As expected from the general action of the enzyme under study, ADP furnishes equimolar amounts of AMP and phosphate (Expt. 1, Table IV). In Expt. 2 evidence is given that ATP undergoes cleavage primarily between the β- and γ-phosphate groups to yield ADP, which is then slowly converted to AMP. Paper chromatography of the reaction mixtures in isobutyric acid–water–concentrated ammonium hydroxide (66:33:1) also indicated that ADP is the initial product of ATP degradation.

Several attempts have been made to determine whether a single enzyme possesses Mg^{++}-pyrophosphatase activity and the Zn^{++}-dependent activities reported here. The data in Table V indicate that one relatively crude and four crystalline pyro-

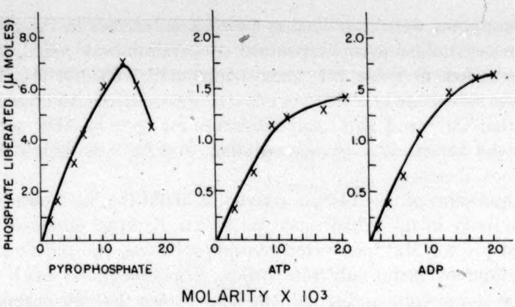

Fig. 3. Phosphate liberation from various substrates as a function of substrate concentration. The usual assay was modified in that 1 μg enzyme was employed with the zinc: pyrophosphate ratio 0.5; 10 μg enzyme with the zinc: ATP ratio 2.0; and 50 μg enzyme with the zinc: ADP ratio 2.0. Incubation, 20 min at 38° with ATP and ADP; 15 min with pyrophosphate.

TABLE IV

LIBERATION OF AMP AND PHOSPHATE BY HYDROLYSIS OF ATP OR ADP

In Expt. 1 the reaction mixture contained 4 μmoles ADP, 4 μmoles zinc acetate, 300 μmoles succinate buffer, pH 5.9, and 100 μg pyrophosphatase in a volume of 3.0 ml. The reaction was stopped by the addition of trichloroacetic acid. In Expt. 2 the reaction mixtures contained 4 μmoles ATP, 8 μmoles zinc acetate, 100 μmoles succinate buffer, pH 5.9, and 10 μg pyrophosphatase in a volume of 3.0 ml. At the intervals indicated individual tubes were heated to stop the reaction.

Expt.	*Substrate*	*Time (min)*	*AMP formed (μmoles)*	*Phosphate formed (μmoles)*
1	ADP	0	0	0
		30	1.59	1.64
2	ATP	10	0.08	1.28
		20	0.14	1.98
		30	0.22	3.32

TABLE V

PYROPHOSPHATASE, ATP-ASE, AND ADP-ASE ACTIVITY OF VARIOUS YEAST INORGANIC PYROPHOSPHATASE PREPARATIONS

The assays were carried out with limiting amounts of enzyme as described in the text, with substrates in these amounts: 50 μmoles Mg^{++} + 2 μmoles pyrophosphate; 4 μmoles Zn^{++} + 2 μmoles pyrophosphate, ATP, or ADP. Incubation, 30 min with ATP or ADP, 15 min with pyrophosphate.

Substrate	*Metal added*	*Specific activity of enzyme (μmoles phosphate liberated/10 μg protein)*				
		Partially purified preparation	*Crystalline preparations*			
			A	*B*	*C*	*D*
Pyrophosphate	Mg^{++}	$3.0 \cdot 10^{-2}$	54	64	85	110
Pyrophosphate	Zn^{++}	$2.3 \cdot 10^{-2}$	43	51	71	98
ATP	Zn^{++}	$1.6 \cdot 10^{-3}$	1.9	2.0	1.7	2.6
ADP	Zn^{++}	$0.2 \cdot 10^{-3}$	0.4	0.4	0.5	0.7

phosphatase preparations possess the same relative activity toward various substrates. In addition, attempts at partial denaturation by exposure to acetone or heating gave no indication for separate ATPase and pyrophosphatase activities (Table VI). One of the crystalline preparations (25 mg/ml in 0.01 M veronal buffer, pH 7.0) exhibited only one peak in the ultracentrifuge during 90 min at 59,000 rev./min at 4°*. After brief centrifugation to remove a trace of denatured protein, another crystalline preparation was submitted to zone electrophoresis in starch gel[11] in sodium borate buffer, pH 8.6, and found to give a single protein band, about 1.5 cm from the origin**. A portion of the protein eluted from this band with 0.1 M Tris–maleate buffer, pH 6.0, had the same relative activity toward ATP (Zn^{++}) and pyrophosphate (Mg^{++}) as before electrophoresis.

TABLE VI

EFFECT OF PARTIAL DENATURATION ON PYROPHOSPHATASE AND ATP-ASE ACTIVITIES

The assays were carried out as indicated in the legend to Table V.

Attempted denaturation procedure	*% activity retained*		
	Mg^{++}-activated pyrophosphatase	*Zn^{++}-activated pyrophosphatase*	*Zn^{++}-activated ATPase*
Incubation with 20% acetone for 4 h at room temperature	100	100	100
Heating at 46° for 5 min	72	76	75
Heating at 46° for 5 min, then at 50° for 5 min	34	35	28
Same as above	18	22	25

DISCUSSION

The results presented indicate that crystalline preparations of yeast inorganic pyrophosphatase attack a number of organic pyrophosphate esters when Mn^{++}, Co^{++}, or Zn^{++} ions are substituted for Mg^{++}. Although the enzymic assays and physical studies described above suggest that a single protein possesses these various activities, it must be emphasized that two or more proteins might not be distinguishable by the methods so far employed.

The following turnover numbers, expressed as moles substrate hydrolyzed per 100,000 g protein per min under the conditions specified, have been calculated for preparation D from the data in Table V: pyrophosphate (Mg^{++}), $3.7 \cdot 10^4$; pyrophosphate (Zn^{++}), $3.3 \cdot 10^4$; ATP (Zn^{++}), $8.7 \cdot 10^2$; and ADP (Zn^{++}), $2.3 \cdot 10^2$. It should be pointed out that the turnover number would have been about 3 times as great if the optimal Zn^{++}: pyrophosphate ratio (0.5) had been used. Determined under slightly different conditions, the turnover number for magnesium-activated pyrophosphatase has been reported[2] as $6 \cdot 10^4$. The rate at which ATP and ADP are attacked and the apparent affinity of the enzyme for these substrates (*cf.* Fig. 3) indicate that the nucleoside pyrophosphatase activity of yeast pyrophosphatase preparations may be physiologically significant.

To our knowledge there have been no previous reports that the substitution of other metal ions for Mg^{++} extends the specificity of a purified inorganic pyrophosphatase. For example, the partially purified inorganic pyrophosphatase from pig brain[12] has an absolute requirement for magnesium ions. Other investigators[2,6] have noted that the activity of pyrophosphatase is greatly influenced by the metal–substrate ratio, as shown also in the present work. Studies on the role of the metal have led to the proposal that magnesium pyrophosphate may serve as the substrate for erythrocyte[13] and rat brain[14] pyrophosphatases. In examining the exchange of [^{18}O]phosphate with water, catalyzed by yeast inorganic pyrophosphatase, COHN[15] has recently obtained evidence that magnesium phosphate may be a substrate in this exchange reaction.

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* Kindly carried out by Dr. W. D. Block.

** Kindly carried out by Dr. R. L. Hunter.

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THE ENZYMIC OXIDATION OF [$16\text{-}^{14}C$]OESTRONE *IN VITRO*

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SUMMARY

Incubation of [$16\text{-}^{14}C$]oestrone with mushroom extracts or a mushroom tyrosinase preparation resulted in the formation of ether-insoluble products in high percentage yields. The nature of these metabolites has been investigated and the evidence points to an initial enzymic oxidation of oestrone to highly reactive *o*-quinonoid derivatives which then combine with protein or undergo alternative reactions. A very strong chemical bond is formed with protein and all attempts to release the oestrone metabolites from it were unsuccessful. Rat-liver slices are also capable of forming ether-insoluble products from oestrone, but only a relatively small fraction of these was found associated with protein. The ether-insoluble compounds showed no marked oestrogenic activity and the significance of protein binding in oestrogen metabolism is discussed.

INTRODUCTION

The conversion of oestrone to water-soluble ether-insoluble products by mammalian liver slices was first demonstrated by SZEGO[1] and later by HEUSGHEM[2]. More recently, JELLINCK[3] obtained these metabolites in high percentage yield on incubating [$16\text{-}^{14}C$]-oestrone with either liver slices or mushroom extracts. The latter system was used because it had been shown by WESTERFELD[4] and GRAUBARD AND PINCUS[5] that oxidases from plant sources were capable of inactivating oestrogens. Since the products formed by liver were found to differ in part from those formed by the mushroom phenol oxidases, it was decided to investigate each of these biochemical reactions and the present paper deals mainly with the results obtained from the relatively simpler plant phenol oxidase system.

MATERIALS AND METHODS

Bovine γ-globulin, crystalline bovine plasma and egg albumin were obtained from the Armour Laboratories. Bacteriological peptone, papain, glycogen, ascorbic acid and all the amino acids were from British Drug Houses, Ltd., glutathione from Distillers Co. (Biochemicals) Ltd., gelatin from Hopkin and Williams Ltd., and the peptides from Hoffman-La Roche & Cie. Casydrol, an enzymic hydrolysate of fat-free whole milk containing approximately equal parts of amino acids with polypeptides, was obtained from Benger Laboratories Ltd. The ether used was free from peroxides and *p*-nitrophenol was recrystallized from aqueous ethanol. All other chemicals were the purest available commercially.

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Radioactive steroids

[16-^{14}C]oestrone (10 μC), purified by chromatography over alumina, was obtained from the Radiochemical Centre, Amersham, Bucks. [16-^{14}C]oestrone methyl ether was prepared from radioactive oestrone (1 mg) by the method of BROWN[6]. The methyl ether was shown to be free from starting material by chromatography with the toluene–propylene glycol system[7], followed by ^{14}C assay of the developed chromatogram. The dried paper was divided into 1-cm segments and each square counted in the end-window counter.

Buffer solution

The incubation medium was phosphate buffer prepared by titrating 0.2 *M* KH_2PO_4 solution with 0.2 *N* NaOH solution to pH 6.8 as measured with a standardized glass electrode. In addition the pH of each mixture was checked with indicator at the beginning and end of the experiment.

Mushroom tyrosinase

A mushroom extract or a mushroom tyrosinase preparation (Worthington Biochemical Corporation) was used. The extract, which was prepared by grinding fresh mushrooms (20 g) with sand under water (25 ml) and centrifuged after straining through muslin, would contain several phenol oxidases.

Measurement of radioactivity

This was determined on polythene planchets (internal diameter 1.5 cm) in a Geiger-Muller counter (G.C.E. EHM2/S) with a thin mica-window (1.7 mg/cm^2) coupled to a scaler and E.H.T. unit (Ekco type N53OD). The samples for plating were either dissolved in ethanol and left to evaporate at room temperature or dried over P_2O_5 at reduced pressure if they were in aqueous solution. Carbon dioxide collected during incubation was precipitated as $BaCO_3$ and this method, as well as the counting procedure at infinite thinness, were as previously described[3]. [^{14}C]Perspex (from the Radiochemical Centre, Amersham) was used for the standard count.

Bioassay

The aqueous medium after extraction with ether was administered to a group of five ovariectomized rats, the procedure of subcutaneous injection and vaginal smearing being the standard one used at this Institute[8]. The median effective dose (ED_{50}) by this method is 1.2 μg oestrone/rat.

Incubation

Unless otherwise stated the solutions (5 ml) were incubated for 5 h in 20 ml glass stoppered test tubes at room temperature. The radioactive oestrone, was dissolved in ethanol (1 mg/ml), and 0.3 ml added to 30 ml of the buffer. Portions (2 ml) of the resulting solution containing 20 μg (3800 counts/min) of the oestrogen were mixed with the enzyme solution (2 ml) and made up to 5 ml with water or with an aqueous solution of the inhibitor or other substance. In this last fraction (1 ml), arginine, cysteine, histidine, and lysine were dissolved as the hydrochloride and the pH re-adjusted to 6.8 with 0.1 *N* NaOH; the ascorbic acid and iodoacetate solutions were also titrated with 0.1 *N* alkali to this pH and the *p*-chloromercuribenzoate solution was prepared by dissolving the acid in *N* NaOH, adding *N* HCl until the pH was 6.8 and then diluting tenfold.

Anaerobic incubations were carried out in Thunberg tubes (25 ml) and they were also used with 5 *N* KOH (1 ml) in the hollow stopper for CO_2 collection.

The final concentration of ethanol in the incubation medium was 0.4 % (v/v).

Extraction of the incubated medium

The medium was extracted twice with equal volumes of ether immediately after incubation and the upper solvent layer removed by teat pipette after each extraction.

The ethereal fraction was evaporated to dryness at about 45° and the residue dissolved in ethanol for assay of radioactivity. The aqueous fraction was plated without further treatment.

Examination of aqueous fractions

Portions (3 ml) of the aqueous solutions were acidified to pH 1–2 with 12 *N* HCl and extracted twice with equal volumes of ether.

Strong acid hydrolysis was carried out by refluxing or by heating in a sealed tube at 150° with 7 *N* HCl for 24 h. Weak acid hydrolysis was performed by refluxing with 2 *N* HCl for 2 h.

Papain (2 mg) activated by HCN (0.05 *N*) was used for the enzymic hydrolysis and dialysis was carried out in Visking dialysis tubing for 24 h against running water, 2 ml of the aqueous fraction being used in both methods.

Protein was precipitated by the addition of 0.125 ml of 6 *N* trichloroacetic acid/ml solution, centrifuged and dissolved in water for assay of radioactivity.

RESULTS AND DISCUSSION

The results (Tables I and II) show that mushroom extracts and a tyrosinase preparation are both capable of attacking oestrone under aerobic conditions with the formation of ether-insoluble metabolites in high percentage yield. Almost complete conversion of oestrone to these products could be obtained by increasing the time of incubation to 24 h and the temperature to 37°.

The discrepancies in total recovery of radioactivity are mainly due to errors introduced in measuring accurately, by pipette, small volumes (0.1 ml) of the concentrated [^{14}C]oestrone solution, and also to the difficulty of correcting samples of the aqueous fraction for self-absorption. Thus the greatest discrepancies often occurred when high yields of water-soluble metabolites were formed and a given sample gave more counts when the protein dissolved in it had been denatured before plating. One minor contributing factor is the counting error (< 5 %).

The reaction was enzymic since virtually all the radioactivity could be removed

TABLE I

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY AFTER INCUBATION OF $[16\text{-}^{14}C]$OESTRONE WITH MUSHROOM EXTRACT

Conditions as described in text.

Experimental conditions	*Percentage of added radioactivity*		
	Aqueous medium after extraction with ether	*Ethereal extract*	*Total recovery*
Normal (incubated for 5 h at room temperature)	29.1	60.3	89.4
	31.9*	40.0	71.9
Mushroom extract boiled	2.0	97.1	99.1
Control (mushroom extract omitted)	< 0.5	100.9	100.9
Anaerobic incubations	1.6	84.5	86.1
	1.9	77.9	79.8
Solution saturated with CO (coal gas)	0.7	—	—
Incubated for 24 h at room temperature	55.0	12.1	67.1
Incubated for 24 h at 37°	79.0	6.2	85.2
KCN (0.01 *N*) added	< 0.5	94.8	94.8
p-nitrophenol (0.01 *M*) added	6.9	72.8	79.7
	9.6	64.0	73.6
Iodoacetate (0.02 *M*) added	30.3	52.3	82.6
$[^{14}C]$oestrone methyl ether substituted for oestrone	1.9	—	—

* CO_2 collected showed no radioactivity.

TABLE II

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY AFTER INCUBATION OF $[16\text{-}^{14}C]$OESTRONE WITH MUSHROOM TYROSINASE

Experimental conditions	*Weight of tyrosinase incubated (mg)*	*Concn. of inhibitor (mM)*	*Percentage of added radioactivity*		
			Aqueous medium after extraction with ether	*Ethereal extract*	*Total recovery*
Normal (5 h at room temperature)*	2	—	72.9**	—	—
			78.6	17.6	96.2
Incubated for 24 h at room temperature	2	—	85.7**	7.6	93.3
Catechol added	2	0.1	87.0	7.5	94.5
			91.3	9.1	100.4
Tyrosinase solution boiled	2	—	0.5	100.1	100.1
$[^{14}C]$oestrone methyl ether substituted for oestrone	2	—	1.7	—	—
p-chloromercuribenzoate added	2	0.1	76.6	16.5	93.1
Ascorbic acid added	2	3.0	2.5	80.3	82.8
Normal (5 h at room temperature)*	1	—	50.9	26.7	77.6
Anaerobic incubation	1	—	4.9	68.7	73.6
p-chloromercuribenzoate added	1	1.0	46.1	29.9	76.0
Iodoacetate added	1	2.5	49.2	26.7	75.9
		5.0	47.3	26.1	73.4
		10.0	47.4	25.6	73.0
Ascorbic acid added	1	1.5	3.2	70.9	74.1
		3.0	2.6	69.4	71.8
p-nitrophenol added	1	5.0	1.1	69.8	70.9
KCN added	1	1.0	1.5	78.9	80.4
Normal (5 h at room temperature)*	0.3	—	21.4	68.0	89.4
Sodium azide added	0.3	10.0	0.5	91.2	91.7
o-phenylenediamine added	0.3	3.7	0.7	98.1	98.8

* Conditions as described in text.

** Oestrone metabolites in aqueous fraction, pooled from 5 incubations, showed no oestrogenic activity at a dose of 12 μg/rat.

from the aqueous medium by ether if the mushroom extract or tyrosinase solution were boiled before incubation.

Effect of biological inhibitors

Substances known to form a complex with copper, such as potassium cyanide, sodium azide, carbon monoxide (Table II), cysteine and glutathione (Table III), acted as powerful inhibitors but iodoacetate and *p*-chloromercuribenzoate, which react with sulphydryl groups, had no effect. Biological reducing agents prevented the formation of ether-insoluble products from oestrone by tyrosinase and *p*-nitrophenol, which can act as a competitive inhibitor also showed this effect. Catechol, however, enhanced the reaction and this compound is known to remove the characteristic induction period of tyrosinase.

These results therefore suggest that the mushroom enzyme acts on oestrone in the same manner as on its normal phenolic substrates with the formation of one or both of its possible *o*-quinonoid derivatives (Fig. 1). Additional support for initial

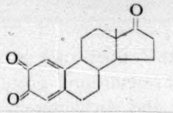
TABLE III

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY AFTER INCUBATION OF [16-^{14}C]OESTRONE WITH MUSHROOM TYROSINASE

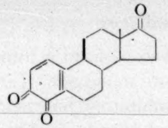
Added substance	*Weight of tyrosinase incubated (mg)*	*Weight of added substance (mg)*	*Percentage of added radioactivity*		
			Aqueous medium after extraction with ether	*Ethereal extract*	*Total recovery*
None	0.4	—	30.4	50.4	90.8
Egg albumin	0.4	25	74.6	17.1	91.7
Bovine serum albumin	0.4	25	74.6	7.7	82.3
Bovine serum albumin (boiled)	0.4	25	42.1	28.8	70.9
Bovine serum albumin (tyrosinase omitted)	—	25	1.0	78.1	79.1
			1.3	82.7	84.0
None	0.3	—	24.5	52.3	76.8
			25.5	53.1	78.6
Bovine serum albumin	0.3	2	53.3	34.1	89.4
Bovine serum albumin	0.3	10	74.5	14.1	88.6
			72.6	14.4	87.0
Bovine serum albumin (boiled)	0.3	10	47.5	37.1	84.6
Bovine serum albumin (added after incubation)	0.3	10	40.7	33.6	74.3
			39.5	48.0	87.5
Bovine gamma globulin	0.3	10	54.8	33.3	88.1
Human serum (0.2 ml)	0.3	—	60.5	30.4	90.9
Gelatin	0.3	10	44.7	39.0	83.7
			39.7	44.3	84.0
Peptone	0.3	10	35.0	43.7	78.7
Glycogen	0.3	10	27.7	49.9	77.6
Glutathione	0.3	10	0.5	93.3	93.8
"Casydrol" protein hydrolysate	0.3	10	13.9	61.9	75.8
None	0.3	—	21.4	68.0	89.4
			20.0	66.9	86.9
Cysteine	0.3	10	4.2	82.1	86.3
			3.3	83.5	86.8
Histidine	0.3	10	7.4	80.0	87.4
			4.4	75.2	79.6
Various amino acids and peptides*	0.3	10	20.9–30.5	—	—

* Alanine, arginine, asparagine, glutamine, glycine, hydroxyproline, leucine, lysine, proline serine, tryptophan, valine, glycylglycine, alanylglycine, leucylglycylglycine.

attack on ring A of oestrone is given on blocking the phenolic group of the oestrogen by methylation since no ether-insoluble metabolites were formed under these conditions.



oestrone-2,3-quinone



oestrone-3,4-quinone

Fig. 1. Possible o-quinonoid derivatives formed during the oxidation of oestrone by tyrosinase.

The *o*-quinones produced as metabolic intermediates are among the most reactive compounds occurring in living matter and the least restricted in respect to the chemical groups with which they can readily interact. However, with a large molecule, such as the *o*-quinone of oestrone, the number of possible reactions would be limited by steric factors and, in the tyrosinase experiment, interaction with sulphydryl groups only, can be ruled out since *p*-chloromercuribenzoate and also iodoacetate in relatively high concentration did not influence the formation of ether-insoluble metabolites.

Effect of protein

Polymerization, or combination with a primary or secondary amino-group of protein in the incubation medium are possibilities and one or more of these reactions may be occurring. The addition of protein greatly increased the amount of ether-insoluble ^{14}C when less than optimum amounts of the mushroom tyrosinase preparation were used and the experimental evidence favours the formation of a strong chemical bond and not just adsorption onto the protein. Thus, even after prolonged hydrolysis with hydrochloric acid, only small amounts of radioactivity could be removed from the aqueous fraction by ether (Table V), whereas this solvent removed all the ^{14}C when radioactive oestrone and albumin were incubated without tyrosinase (Table III). Conditions under which proteins are hydrolysed also resulted in the destruction of the steroid and an attempt to release the bound ^{14}C by enzymic hydrolysis was unsuccessful.

The specificity of the reaction with protein was low, since bovine serum albumin or globulin, egg albumin, human serum proteins, and mushroom tyrosinase were all capable of interacting with oestrone metabolites, but gelatin and denatured albumin were not so effective (Table III). In addition, less ^{14}C became bound to protein if the latter was only added at the end of the incubation period, suggesting that a slower alternative reaction was capable of occurring. This could be a polymerization requiring further oxidation of the quinonoid derivatives of oestrone, which otherwise would rapidly combine with amino or other groups.

The findings (Table IV) that much of the ether-insoluble ^{14}C was indiffusible, especially when albumin had been added to the incubation mixture, and also that it accompanied the precipitate formed on adding trichloroacetic acid, is further evidence for the interaction of oestrone metabolites with protein.

Effect of amino acids and peptides

The results (Table III) of the experiments with added amino acids and peptides show that cysteine and glutathione, by virtue of their reducing and metal-binding

TABLE IV

EXAMINATION OF THE ETHER-EXTRACTED AQUEOUS FRACTION AFTER INCUBATION OF [16-^{14}C] OESTRONE WITH MUSHROOM TYROSINASE AND RAT LIVER SLICES

Enzyme	Added material	Percentage ^{14}C of aqueous fraction		
		Trichloroacetic acid precipitate	*Trichloroacetic acid supernatant*	*Indiffusible fraction after dialysis*
Tyrosinase*	—	62.8	35.9	61.9
Liver*	—	14.2	76.4	9.8
	—	43.1	52.6	43.4
Tyrosinase	Bovine serum albumin (10 mg)	76.3	18.2	78.5
	Bovine serum albumin (10 mg)	—	—	90.3
	Human serum (0.2 ml)	—	—	89.4
	—	—	—	46.2
	—	—	—	71.9**

* Bovine serum albumin (5 mg) added before precipitation with trichloroacetic acid.
** Ether-extracted aqueous fraction incubated with papain for 24 h at 37° before dialysis.

TABLE V

EXAMINATION OF THE ETHER-EXTRACTED AQUEOUS FRACTION AFTER INCUBATION OF [16-^{14}C]OESTRONE WITH MUSHROOM TYROSINASE

Treatment	Percentage ^{14}C of aqueous fraction		
	Aqueous medium after extraction with ether	*Ethereal extract*	*Total recovery*
Extracted with ether	98.2	1.8	100.0
Acidified and extracted with ether	79.5	8.9	88.4
Weak acid hydrolysis	69.3	17.3	86.6
Strong acid hydrolysis	45.9	3.2	49.1
Strong acid hydrolysis (in sealed tube at 150°)	17.9	6.4	24.3

properties, inhibited the tyrosinase reaction, and histidine was also found to decrease the amount of ^{14}C not removed from the aqueous solution by ether; but none of the other amino acids or peptides tested had any marked effect. These results could imply that amino acids which are known to combine with simple quinones[9,10] do not react with the *o*-quinonoid derivatives of oestrone or, more probably, that the latter products are formed, but still show partition in favour of the organic solvent. The solubility in water would be expected to increase with increasing molecular weight of the hydrophilic adduct, which would account for the results obtained with proteins and peptone. This is not a non-specific interaction with any compound of high molecular weight, since glycogen had no effect. The inhibition produced by a protein hydrolysate, such as Casydrol, containing a mixture of amino acids and peptides, is probably due to the presence of cysteine and histidine.

Effect of o-*phenylenediamine*

Quinones also react with amines and *o*-phenylenediamine would be expected to trap the *o*-quinonoid oestrogen metabolites as ether-extractable phenazine derivatives. No water-soluble ^{14}C was observed after incubation of [^{14}C]oestrone with tyrosinase in the presence of *o*-phenylenediamine (Table II), which supports the proposed reaction unless low concentrations (3.7 m*M*) of the diamine inhibit the enzyme. However, there was no inhibition when similar quantities of this reagent were used[11] to estimate the phenol oxidase activity of potatoes.

The CO_2 evolved during incubation, as expected, contained no ^{14}C, suggesting that ring D of oestrone was intact and the ether-insoluble metabolites showed no oestrogenic activity at more than 10 times the median effective dose of oestrone.

Incubation with rat-liver slices]

Since mammalian liver slices are also capable of converting oestrone to ether-insoluble products and since RIEGEL AND MUELLER[12] have described the formation of protein-bound metabolites of oestradiol by rat-liver homogenates, it was decided to investigate whether binding with soluble protein could explain the former results. The incubation, extraction and separation into fractions was as previously described[3], but it was found that only a relatively small portion (10–15 %) of the ether-insoluble radioactivity formed by liver could be precipitated with trichloroacetic acid, or would not diffuse on dialysis (Table IV) when, under similar conditions with tyrosinase, most of the ^{14}C became associated with protein. It could be that the quinonoid metabolites are taken up by the insoluble proteins on the surface of the slices, which would explain the adsorption of ^{14}C observed in the earlier experiments[3], but it still does not account for the remaining 85–90 % of the radioactivity in the ether-extracted aqueous medium. The organized system of enzymes in liver slices is probably capable of producing more extensive changes in the oestrone molecule than either mushroom extracts or tyrosinase and there is evidence that acidic products are formed, presumably by cleavage of the aromatic ring[3].

ZILLIG AND MUELLER[13], using rat-liver microsomes, have studied the interaction of protein with oestradiol derivatives and have excluded positions 2 and 4 from being involved in the binding process, because substitution of one or other of these positions on the steroid with fluorine did not affect combination with protein. Since 2,4-difluoro-oestradiol was not tested, one of the two possible *o*-quinonoid derivatives of oestradiol could still have been formed in each case. However, HECKER AND MUELLER[14] have isolated a *p*-quinonoid derivative after incubating tetrahydro-2-naphthol with rat liver microsomes, so that the formation of a similar oestrone metabolite cannot be excluded, although the isolation of 2-methoxyoestrone[15] from human pregnancy urine supports conversion of oestrone to an *o*-quinone.

The formation of ether-insoluble polymers from oestrogens by liver must also be considered and the fraction associated with protein, though relatively small, may have an important role in the physiological action of oestrone and may be produced in greater amounts *in vivo*.

Protein-binding could also have some significance in the carcinogenic action of oestrone[16], since many compounds with carcinogenic activity have been found to bind firmly to cellular proteins[17] at the site of tumour formation.

Finally, the possibility exists that, *in vivo*, oestrogen metabolites are excreted in microgram amounts as biologically inert polypeptide conjugates, which are stable under conditions used to hydrolyse sulphates and glucuronides and which would not be extracted from urine by organic solvents. This type of compound, and also acid or polymerized products, may account for the ether-insoluble radioactivity of hydrolysed urine observed[18,19] in rats treated with [^{14}C]oestrone and in human patients injected with [^{14}C]oestrogens.

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Biochim. Biophys. Acta, 41 (1960) 37–45

METABOLISM OF PHOSPHOLIPIDS*

II. ISOLATION AND PROPERTIES OF PHOSPHATIDIC ACID FROM MAMMALIAN LIVER

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SUMMARY

1. A procedure is described for the isolation of phosphatidic acid from mammalian liver (ox, pig and rat).

2. Analytical data are presented identifying this compound as phosphatidic acid.

3. Between 80 and 90 % of the fatty acids of phosphatidic acid are unsaturated, the majority being linoleic acid.

4. The biochemical significance of phosphatidic acid is discussed.

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[*] For Part I, see ref. 3.

INTRODUCTION

Recent investigations on the biosynthesis of phospholipids showed that phosphatidic acid might be involved as an intermediate[1–3]. Although KORNBERG AND PRICER[4] had demonstrated the biosynthesis of phosphatidic acid in particulate preparations of rat liver, the isolation of this phospholipid from animal tissue had not been achieved. DAWSON[5] and MARINETTI AND STOTZ[6] stated, moreover, that they were unable to detect phosphatidic acid *in vivo* and MARINETTI and co-workers[7] described the occurrence of a compound similar to phosphatidic acid but which differed from it in that it had a higher molar ratio of P/ester than would be expected for phosphatidic acid.

The work of HOKIN AND HOKIN[8] indicated, however, that phosphatidic acid might occur in animal tissues. HAWTHORNE AND HÜBSCHER[9] concluded from time studies on mild alkaline hydrolysis of total phospholipids, that the small amounts of glycerophosphate isolated had most probably been derived from phosphatidic acid. Finally GRAY AND MACFARLANE[10] mentioned, in their work on polyglycerophosphatides of the ox heart, a minute constituent which might have been phosphatidic acid.

The present work describes a simple procedure for the isolation of phosphatidic acid from liver and gives analytical data on the compound isolated. A preliminary report has previously been published[11].

MATERIALS AND METHODS

Most of the methods used were described previously[9,12]. Silicic acid (100 mesh, for chromatography) was used as obtained from Mallinkrodt Chemical Works, St. Louis, New York and Montreal. Chloroform was of Analar grade and contained about 1 % of ethyl alcohol as stabiliser. Throughout this work, the concentrations of methanol in chloroform quoted exclude that of the alcohol present as stabiliser.

Glycerol was determined by the chromotropic acid method as described by BURTON[13]. The separation of glycerol on Celite was replaced by the following method. An aliquot of the hydrolysate (6 N HCl; 40 to 48 h at 110°) was diluted to give a final volume of 10 ml. 1 g of dry charcoal (Norit A) was added and enough of dry Bio-Deminrolit mixed bed resin to bring the pH to 7. The mixture was stirred for 3 min, left standing for 30 min and then filtered. An aliquot of the filtrate was analysed for glycerol. As a standard, authentic α-glycerophosphate was taken through the same procedure.

DL-α-glycerophosphate was synthesised by the method of BAER[14]. The barium salt contained 9.95 % P (theory 10.07 % P of the anhydrous salt). Upon treatment of the barium salt with periodic acid[13], 1.05 moles of formaldehyde were liberated per mole of P. Further indication that all of the compound was in the α-form, was obtained by chromatography of the free acid in n-propanol–concentrated ammonia–water (5:4:1, v/v). In this system, the ester prepared moved as a single spot with an R_P value of 1.43, β-glycerophosphate having an R_P value of 1.57 (R_P of phosphoric acid = 1.00).

Esterified fatty acids were determined according to STERN AND SHAPIRO[15], and the ethylenic linkages of long chain fatty acids by the method of TRAPPE[16]. Aldehydes were determined as described by Gray and Macfarlane[10]. Palmitaldehyde dimethylacetal was used as a standard, 10 μg gave an O.D. of 0.16 at 546 mμ (1 cm cell).

Phosphatidic acid was prepared from ovolecithin which had previously been purified on alumina[17]. Cabbage-leaf phospholipase D was obtained from Savoy cabbage as outlined by Davidson and Long[18] and used for the hydrolysis of lecithin under conditions recommended by the same authors. The phosphatidic acid was isolated from the reaction mixture and prepared as the sodium salt as described by Kates[19].

For the preparation of the methylesters of the long chain fatty acids of phosphatidic acid and total phospholipids, the preparations were hydrolysed under mild alkaline conditions for 15 min[9]. After adjustment of the pH of the hydrolysate to about 2, the fatty acids were quantitatively extracted with ether. The combined ether extracts were taken to dryness, and the dry residue was refluxed for 1 h in a few ml of 1 % HCl in methanol. The methyl esters were stored in petroleum ether (40–60°) under nitrogen in a sealed tube at 4°.

EXPERIMENTS AND RESULTS

Chromatographic behaviour of phosphatidic acid from lecithin

In Fig. 1, the chromatography (silicic acid column) of phosphatidic acid prepared from ovolecithin is shown. Relatively low concentrations of methanol in chloroform (3 and 6% respectively) brought about the elution of over 50 % of the phosphatidic acid applied. Since the lowest concentrations of methanol in chloroform quoted in the literature for the elution of phosphatidyl inositol[20] or of phosphatidyl serine and phosphatidyl ethanolamine[21] from silicic acid columns (Mallinkrodt) were 9 to 17 and 14.5 % respectively, it seemed possible to separate phosphatidic acid from the other glycerophosphatides on silicic acid. The only other glycerophosphatide which is eluted with similarly low concentrations of methanol in chloroform (about 10 %)

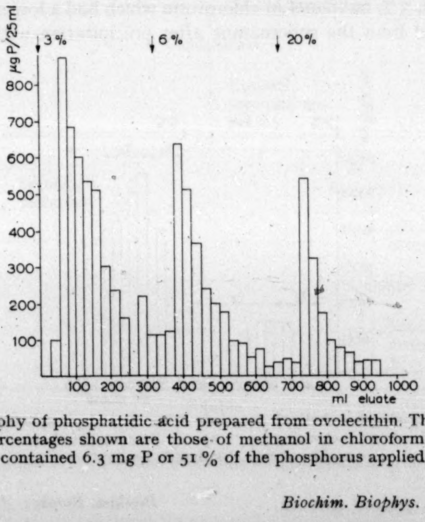

Fig. 1. Chromatography of phosphatidic acid prepared from ovolecithin. The column dimensions were 2 × 25 cm. Percentages shown are those of methanol in chloroform. The first two peaks contained 6.3 mg P or 51 % of the phosphorus applied.

is the polyglycerophosphatide from heart tissues[10]. This phospholipid, however, differs sufficiently in its composition to be distinguished analytically from phosphatidic acid.

Preliminary experiments with phosphatidic acid from ox liver

A portion of an ox liver was minced and partially dried with acetone (1 l of minced tissue +2.5 l of ice-cold acetone). The acetone was removed by filtration through cheesecloth. The partially dried tissue was then extracted with 10 volumes of chloroform–methanol (2:1, v/v) at room temperature overnight. This treatment ensures complete extraction of phospholipids from tissues[22]. The extract was taken to a small volume under nitrogen and reduced pressure and dried over anhydrous sodium sulphate. As phosphatidic acid, if present at all, could be expected in only minute amounts, the concentrated and dried solution of phospholipids was treated with alcohol in the hope of achieving some purification. The chloroform concentrate was treated with 10 volumes of absolute alcohol and left for 16 h, at 4°, the final pH of the solution being about 5. The precipitate was collected by filtration (Fraction A). The pH of the filtrate was adjusted to 8.5 by the addition of a few ml of 1 *N* NaOH. After leaving the solution for 16 h at 4°, a second precipitate (Fraction B) was collected. The filtrate was taken to dryness under nitrogen and reduced pressure and dissolved in chloroform (Fraction C). All fractions were applied to silicic acid columns and elution was carried out as indicated in Fig. 2 for Fraction A. Each of the two peaks obtained with 3 and 6% respectively of methanol in chloroform, was split into two portions (I_1, I_2, II_1 and II_2) and analysed for phosphate, nitrogen and fatty acid esters. The molar ratios of ester P varied only slightly in the different fractions and were between 1.98 and 2.01 per mole of phosphate, whereas the molar ratios of P/N showed marked differences. They were 1:0.09, 1:0.08, 1:0.90 and 1:1.10 for fraction I_1, I_2, II_1 and II_2 respectively, indicating a rather sharp separation of a phospholipid containing very little nitrogenous material. Only fractions A and B gave a peak with 3% methanol in chloroform which had a low molar ratio of P/N. Fraction C, derived from the supernatant after precipitation with alcohol, did not

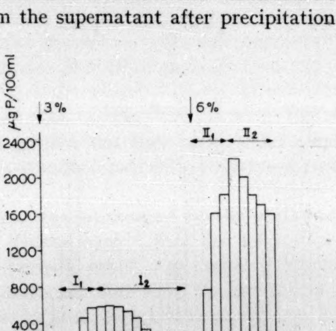

Fig. 2. Separation of crude phospholipid (fraction A). 90 mg of phospholipid phosphorus were applied to a silicic acid column measuring 3.5 × 23 cm. Percentages shown are those of methanol in chloroform. For details see text.

contain phospholipid which could be eluted with such a low concentration of methanol and the first fraction eluted contained considerable amounts of nitrogen.

Procedure for the preparation of highly purified phosphatidic acid

The scheme shown in Fig. 3 summarises the various steps in the preparation of a relatively pure sample of phosphatidic acid.

The freshly obtained liver was minced and partially dried with three volumes (w/v) of ice-cold acetone for 4 h at 0°. The tissue was then extracted twice with 5 volumes of chloroform–methanol (2:1, v/v) for periods of 10 to 20 h at room temperature. The combined extracts were taken to a small volume (about 500 ml) under nitrogen and reduced pressure. The concentrate contained considerable amounts of water and was therefore dried overnight over anhydrous sodium sulphate. The sodium sulphate was removed by filtration and washed with about 100 ml of chloroform. To the combined filtrates, 10 volumes of alcohol were added, and the pH of the solution was

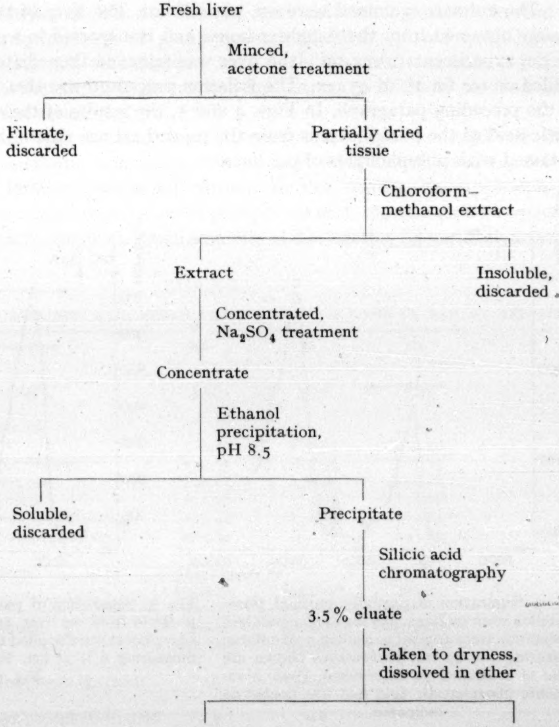
Fresh liver
Minced, acetone treatment
Filtrate, discarded
Partially dried tissue
Chloroform–methanol extract
Extract
Insoluble, discarded
Concentrated. Na_2SO_4 treatment
Concentrate
Ethanol precipitation, pH 8.5
Soluble, discarded
Precipitate
Silicic acid chromatography
3.5 % eluate
Taken to dryness, dissolved in ether
Soluble
Insoluble, discarded

Fig. 3. Diagram of the isolation procedure for phosphatidic acid from liver.

adjusted to 8.5 by the addition of 6 *N* NaOH. The mixture was left for at least 16 h at 0°. The precipitate (containing between 16 and 24 % of the phosphate originally extracted with chloroform–methanol) was collected, dissolved in chloroform and applied to a silicic acid column. This was made up by mixing silicic acid with half its weight of Hyflo Super-Cel. 1 mg of phospholipid P was loaded on to 1.1 g of silicic acid. After applying the phospholipid, the column was washed with 10 to 15 column volumes of chloroform in order to remove coloured material and lipids other than phospholipids. Elution was started with 3.5 % of methanol in chloroform. In some experiments, elution was carried out with 4.5 % of methanol in chloroform without decreasing the purity of the preparation. The phospholipid eluted with this concentration was taken to dryness under nitrogen and at reduced pressure. The dry material was dissolved in ether and the insoluble part removed by centrifugation. The sodium salt of phosphatidic acid was prepared as described by KATES[19].

Isolation and properties of phosphatidic acid from the liver of various animals

The animals examined were ox, pig and rat. The liver of the ox and pig were freshly obtained from the slaughter house and transported in ice to the laboratory. In the experiments using rats, the liver was removed immediately after death and chilled on ice for 15 to 45 min. The isolation procedure was then started as outlined in the preceding paragraph. In Figs. 4 and 5, the results of the chromatography on silicic acid of the phospholipids from the ox and rat are shown. Similar results were obtained with phospholipids of pig liver.

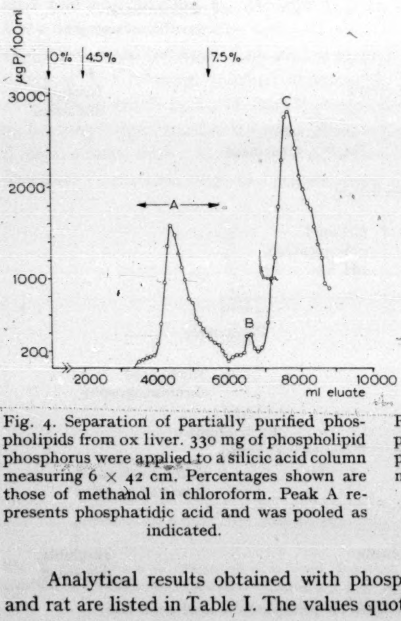

Fig. 4. Separation of partially purified phospholipids from ox liver. 330 mg of phospholipid phosphorus were applied to a silicic acid column measuring 6 × 42 cm. Percentages shown are those of methanol in chloroform. Peak A represents phosphatidic acid and was pooled as indicated.

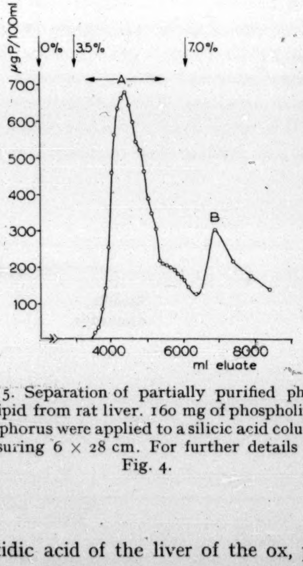

Fig. 5. Separation of partially purified phospholipid from rat liver. 160 mg of phospholipid phosphorus were applied to a silicic acid column measuring 6 × 28 cm. For further details see Fig. 4.

Analytical results obtained with phosphatidic acid of the liver of the ox, pig and rat are listed in Table I. The values quoted for aldehydes (6 to 7 % phosphatidal compounds for the preparations from the ox and pig) are slightly higher than the values given in the literature for total phospholipids of the liver. RAPPORT AND LERNER[23] quoted 3 % plasmalogen content in total phospholipids of rat liver. In the present work, total phospholipids of pig liver were compared with the phosphatidic acid prepared from this tissue. The molar ratio of P aldehyde was 1:0.04 for total phospholipid as compared with 1:0.06. The result obtained with phosphatidic acid from rat liver is too high due to interference by small amounts of silicic acid eluted only in this preparation from the silicic acid column. Interference by silicic acid in the determination of aldehydes using Fuchsin reagent has recently been reported[24]. Glycerol was determined qualitatively by paper chromatography of the acid hydrolysate[12] and quantitatively by the periodic acid–chromotropic acid method. Ethylenic linkages were determined in A by the sodium bromide–bromine method of TRAPPE[16], whereas in B they were calculated from the relative percentages of unsaturated fatty acids present in the total fatty acids of phosphatidic acid as determined by gas-phase chromatography of the methyl esters (see also Table II).

Serine, ethanolamine, choline and inositol were detected by paper chromatography of the acid hydrolysate of phosphatidic acid. Although no quantitative determinations of serine and ethanolamine were done, the small amounts detected might correspond to the nitrogen determined by the micro-Kjeldahl method and mentioned under molar ratios of P/N.

Glycerophosphate was isolated from the aqueous phase of mild alkaline hydrolysates of phosphatidic acid using Nalcite SAR resin and the sodium tetraborate–ammonium formate system for elution. In this system, glycerophosphate is well separated from other monoesters of phosphoric acid, diesters and phosphoric acid[12]. In Fig. 6, peak A represents small amounts of diesters (6.5 %) and B represents

TABLE I

ANALYTICAL RESULTS OBTAINED WITH PHOSPHATIDIC ACID FROM LIVER OF RAT, OX AND PIG

Determinations	*Rat*	*Ox*	*Pig*
% P by weight*	3.66	3.76	3.93
Molar ratio of			
P/N	1:0.02	1:0.07	1:0.10
P/Ester linkage	1:1.91	1:1.93	1:1.83
P/Aldehyde	1:0.15	1:0.07	1:0.06
P/Glycerol	1:1.01	1:1.03	1:1.06
P/Ethylenic linkage A	1:3.46	1:2.93	1:3.84
P/Ethylenic linkage B	1:3.32	1:2.82	1:3.51
Molisch test	negative	—	negative
Inositol	negative	negative	trace
Serine	trace	present	present
Ethanolamine	trace	present	present
Choline	negative	negative	negative
Glycerophosphate % yield	85	>80	83
Percentage phosphatidic acid in total phospholipids	1.5	0.8	0.7

* The theoretical content of P is 4.16 % assuming an average molecular weight of 744.

glycerophosphate (85 %). A small amount of a phosphoric acid ester (6.8 %) was eluted when the column was washed with 2 *N* ammonium formate. An analysis of this last compound was not carried out. Similar preparations and yields of glycerophosphate were obtained with phosphatidic acid from the liver of the ox and pig. The yields of phosphatidic acid expressed as per cent of the total phospholipids originally extracted varied slightly from animal to animal. It is interesting to note that the highest yield was obtained with rat liver where the greatest precaution was taken to prevent post mortem changes. The figures quoted in Table I are, however, only minimum values referring to the amount of phosphatidic acid actually isolated.

The percentages of different fatty acids present in the various preparations of phosphatidic acid are presented in Table II. To confirm the relatively high percentages

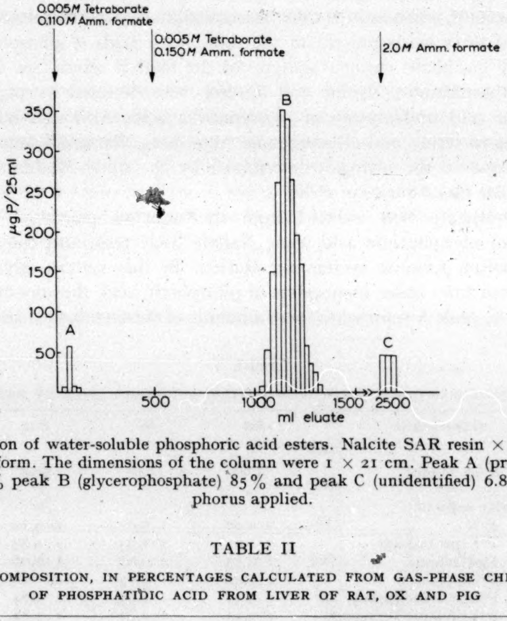

Fig. 6. Separation of water-soluble phosphoric acid esters. Nalcite SAR resin × 10, (100 to 200 mesh) formate form. The dimensions of the column were 1 × 21 cm. Peak A (probably diesters) represents 6.5 % peak B (glycerophosphate) 85 % and peak C (unidentified) 6.8 % of the phosphorus applied.

TABLE II

FATTY ACID COMPOSITION, IN PERCENTAGES CALCULATED FROM GAS-PHASE CHROMATOGRAMS, OF PHOSPHATIDIC ACID FROM LIVER OF RAT, OX AND PIG

Fatty acid	*Phosphatidic acid from liver of*		
	Rat	*Ox*	*Pig*
Palmitic acid	2.6	3.5	1.5
Palmitoleic acid	2.0	5.0	1.3
Stearic acid	1.9	3.6	2.7
Oleic acid	17.6	15.0	9.7
Linoleic acid	72.0	60.5	77.6
Linolenic acid	0.7	—	2.3
C_{20}–C_{22} acids	3.2	8.0	4.9
Unidentified (shorter chain acids)	—	4.4	—

of unsaturated fatty acids, a portion of the methyl esters was hydrogenated using platinum oxide as catalyst. The C_{16} and C_{18} unsaturated fatty acids were thereby completely converted to palmitic and stearic acid respectively. The fatty acids listed under C_{20}-C_{22} acids were only tentatively identified by their position on the chromatogram. Hydrogenation of the methyl esters showed that part of these acids were unsaturated.

DISCUSSION

The present investigation gives evidence that phosphatidic acid in fact occurs in the liver of the ox, pig and rat. 6 to 7 % of this phospholipid seems to be present as plasmalogen.

KORNBERG AND PRICER[4] have described a particulate preparation from rat liver which carries out the biosynthesis of phosphatidic acid from L-α-glycerophosphate and activated long chain fatty acids. HOKIN AND HOKIN[25] suggested an alternative pathway occurring in brain tissue. Here, phosphatidic acid is formed by phosphorylation of D-α,β-diglyceride at the expense of ATP. Furthermore, a partially purified enzyme has been prepared from liver[26] which hydrolyses phosphatidic acid to phosphoric acid and diglyceride. More recently it was suggested that phosphatidic acid might also arise by enzymic breakdown of phospholipids liberating choline and serine from phosphatidyl choline and phosphatidyl serine respectively[27].

The remarkably high percentage of unsaturated fatty acids described in the present investigation is of considerable interest. Oleic acid and linoleic acid corresponded to 75 to 89 %, whereas palmitic and stearic acid amounted to only 4 to 7 % of the total fatty acids. This high degree of unsaturation is in contrast to the distribution of fatty acids in total phospholipids of the liver of various animals, where stearic and palmitic acid represent 30 to 47 % of the total fatty acids[28–30]. It is also difficult to reconcile the present results with the positional asymmetry of fatty acids in lecithins suggested by HANAHAN[31], who postulated an unsaturated fatty acid in the α-position only. Although lecithin belongs to a different group of glycerophosphatides, the two compounds are related biosynthetically in that phosphatidic acid is the precursor of lecithin[1]. Phosphatidic acid is also thought to be the precursor of glycerides[1], which are again more saturated than the phosphatidic acid isolated.

It is known that the early fractions of any phospholipid peak eluted from a silicic acid column with methanol in chloroform contain more unsaturated fatty acids than the later fractions of the same peak[24,32]. It might be possible that the use of only 3.5 % methanol in chloroform would selectively elute the highly unsaturated phosphatidic acid. There is, however, no evidence that appreciable amounts of phosphatidic acid are eluted with higher concentrations of methanol in chloroform when crude phospholipid mixtures are separated on silicic acid columns (see RESULTS). The results of DITTMER AND HANAHAN[33] would support this view. These authors separated phospholipids on silicic acid columns using increasing amounts of methanol in chloroform. The partially purified phospholipids were analysed for their fatty acid composition and there was no increase in unsaturation of the fatty acids in the peaks eluted with higher amounts of methanol in chloroform. It seems, therefore, that the preparation described is a representative sample of the naturally occurring phosphatidic acid.

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ON THE ROLE OF LONG-CHAIN ALDEHYDES IN THE LIGHT REACTION IN *PHOTOBACTERIUM PHOSPHOREUM* ENZYME PREPARATIONS

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SUMMARY

(1) Active luciferase–DPNH-oxidase preparations from *Photobacterium phosphoreum* generally contain some aldehyde-attacking enzyme, probably ADH. Under the experimental conditions applied this enzyme appears to attack decanal, but not palmital.

(2) The presence of long-chain aldehydes in the enzyme preparations could be demonstrated. It was not possible to establish with certainty that the substance that is active in the light reaction catalyzed by enzyme preparations without added aldehyde is a long-chain aldehyde.

(3) There is no experimental evidence for the conversion of palmital in the light reaction.

(4) Extraction of enzyme preparations of *Ph. phosphoreum* with iso-octane increases the light reaction; it seems probable that an inhibitor of luciferase is removed. Palmital, added to the enzyme preparations, is only slightly extracted by iso-octane.

(5) The extent of the light reaction in the enzyme preparations extracted with iso-octane depends on the sequence of addition of palmital, DPNH and FMN; the highest reaction is obtained if palmital is added first. The effect is influenced by aldehyde- and FMN concentrations; it is less conspicuous in non-extracted preparations.

(6) The experimental facts can be explained if it is assumed that palmital (or a long-chain aldehyde very similar to palmital) serves as a binding agent between the protein and FMNH. The possibility of a second, direct attachment of FMNH to the protein is discussed.

INTRODUCTION

In 1953 STREHLER AND CORMIER[1] reported that kidney cortex extract could increase the light reaction in enzyme preparations of luminous bacteria. The active substance proved to be palmital[2]. STREHLER AND CORMIER[1] found that heat-precipitable

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Abbreviations: DPN(H), (reduced) diphosphopyridine-nucleotide; FMN(H), (reduced) flavin mononucleotide; ADH, alcohol dehydrogenase.

fractions obtained from *Achromobacter fischeri* are active in promoting the light reaction of preparations of luminous bacteria. It has not been demonstrated, however, that this activation is due to the presence of long-chain aldehydes in these fractions.

Besides palmital, other long-chain aldehydes (7 or more C atoms) were found to be active in this respect[2]; since it is difficult to obtain palmital, most experiments were done with decanal (C 10).

CORMIER AND TOTTER[3] summarize the events that lead to light emission in bacteria as follows:

$$\mathrm{DPNH} + \mathrm{FMN} \xrightarrow{\text{DPNH-oxidase}} \mathrm{FMNH} + \mathrm{DPN}$$

$$\mathrm{RCHO} + \mathrm{O_2} + \mathrm{FMNH} \xrightarrow{\text{luciferase-FMN}} \mathrm{RCOOH}\ (?) + \mathrm{FMN} + \mathrm{H_2O} + \text{light}$$

It will be shown in this paper that the conversion of the long-chain aldehyde cannot as yet be considered to be definitely proved.

The experiments described here were carried out in an attempt to throw some more light on the role of long-chain aldehydes in the light reaction. Further, an attempt was made to check the assumption that the active substance present in luminous bacteria is a long-chain aldehyde.

MATERIAL AND METHODS

Photobacterium phosphoreum was cultivated in a shaking apparatus at about 17° in a liquid medium containing 1 % peptone (Difco "Bacto"), 3 % NaCl and 0.3 % glycerol in tap water at pH 7.3–7.5. The enzyme preparations were made following the method of McELROY *et al.*[4]. Except where otherwise stated, the HCl precipitate of the supernatant of lysed cells, dissolved in 0.1 M phosphate buffer pH 7.0, was used. No DPNH-oxidase was added. The light reaction was evoked by adding 0.5 mg DPNH (dissolved in 0.5 ml 0.1 M phosphate buffer pH 7.0), 0.2 ml FMN solution (0.001 %) and 0.01–0.025 ml palmital solution (a freshly made, mostly saturated solution in methanol, diluted as indicated in the experiments) to 1 ml enzyme preparation. In the experiments with decanal a saturated decanal solution in 0.1 M phosphate buffer pH 7.0 was used.

Palmital was prepared from the corresponding acid chloride according to ROSENMUND[5].

Extraction with iso-octane was performed at room temperature by shaking the enzyme preparation for 2 min with an equal volume of iso-octane; after centrifugation for 10 min the two layers were separated.

The light reaction proceeded in a cuvette cooled with tap water (resulting in a temperature of the reaction mixture of about 17°). Two preparations could be measured alternately in one experiment. The light was measured in a quantum counter, operating with a liquid-air-cooled photo-multiplier (*cf.* SMIT *et al.*[6]). The individual estimations were accurate to within approximately 10 %.

DPNH-oxidase activity was determined by measuring spectrophotometrically the decrease of absorption at 340 mμ in the same reaction mixture as was used for the light reaction.

ADH activity was measured in a similar way, using acetaldehyde as a substrate and omitting FMN.

EXPERIMENTS AND CONCLUSIONS

A. The conversion of decanal and palmital. The identity of the active factor in the light reaction

In order to ensure a relatively durable light reaction of enzyme preparations, at least two enzymes should be present: DPNH-oxidase (for a constant production of FMNH from FMN) and luciferase (for the light reaction proper), (*cf.* TOTTER AND CORMIER[7]). In thoroughly purified luciferase preparations DPNH-oxidase is absent. Therefore the best reaction is obtained with relatively impure enzyme preparations. We therefore used HCl precipitates of extracts of luminous bacteria. However, the presence of ADH (which catalyzes a reduction of aldehyde) in these preparations is a disadvantage. Even in purified but still active enzyme preparations (fractionated with $(NH_4)_2SO_4$) some ADH activity could be detected. Although ADH is most active towards short-chain aldehydes (RACKER[8]), it also appears to use decanal as a substrate (TOTTER AND CORMIER[7]).

In our preparations, preincubation with decanal led to a decreased light reaction. This effect is much more pronounced if DPNH is present during the preincubation time (the light reaction being initiated with FMN afterwards). To a lesser extent this effect was observed in preparations purified by $(NH_4)_2SO_4$ fractionation; it is probably due to the presence of ADH in the preparations.

The preincubation effect was not observed if palmital was used instead of decanal (Table I). Evidently the palmital is not attacked in the presence of DPNH by enzyme systems other than that active in the light reaction.

TABLE I

THE INFLUENCE OF THE DURATION OF PRE-INCUBATION WITH PALMITAL AND DECANAL ON THE LIGHT REACTION

		Light reaction (counts/$^{7}/_{8}$ min)			
Expt. No.	*Enzyme preparation*	*Incubation with palmital + DPNH*		*Incubation with decanal + DPNH*	
		1 min	*11 min*	*1 min*	*11 min*
1	12–6	231	225	927	597
2	29–5	108	109	179	130
3	12–6	160	158	538	385

Reaction mixture: 1 ml enzyme, 0.5 mg DPNH in 0.5 ml phosphate buffer pH 7.0, 0.2 ml FMN 0.001 %, 0.01 ml palmital solution 1:20, 0.1 ml (Expts. 1 and 2) or 0.05 ml (Expt. 3) decanal solution. Initiation of the reaction with FMN, measurement 4 min after the addition of FMN.

This finding may explain the difference of the decay curves of the light reactions when using either decanal or palmital (Fig. 1a). The decay of the "decanal curve" is probably due to the conversion of decanal, independent of the light reaction.

The curve of the light reaction without addition of a long-chain aldehyde resembles the curve of the reaction with palmital (Fig. 1b). If the unknown substance essential for the light reaction in the original enzyme preparation were an aldehyde, this resemblance might indicate that its chain length is of the order of that of palmital.

We tried to demonstrate chemically the presence of long-chain aldehydes in the enzyme preparations. A preparation of about 12 g of bacteria (wet weight) was extracted with methanol–chloroform, following the method of Folch[9]. In the extract, the long-chain aldehydes were determined according to Wittenberg *et al.*[10]. The amount of aldehyde proved to be 10–20 times the amount that might be expected with regard to the light reaction. Probably the preparations contain plasmalogens (acetal phospholipids). It will be shown in section B that extraction of palmital from luciferase may give rise to complications. Therefore, these experiments do not prove that the long-chain aldehyde found in the preparations participates in the light reaction.

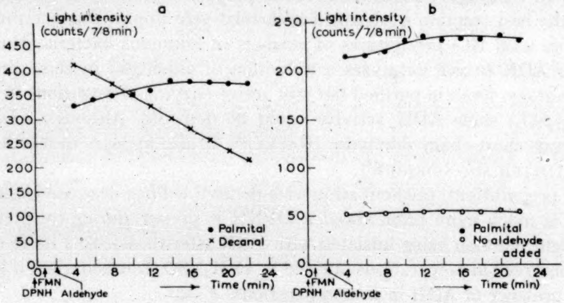


Fig. 1. The light reaction with palmital, decanal and without added aldehyde.

Since, apparently, palmital is no substrate for bacterial ADH, it seemed worth while to consider the question of the conversion of aldehyde during the light reaction once more. McElroy and Green[11] found that the light emitted by bacterial enzyme preparations is directly dependent upon aldehyde concentration. This they interpreted as indicating an utilization of aldehyde in the light reaction. However, it may be objected that the reaction velocity would also, to a certain degree, depend on aldehyde concentration if aldehyde were acting as a catalyst.

The subject has been recently dealt with by Cormier and Totter[3], who estimated the quantum efficiency of decanal in the light reaction. However, their preparations were not highly purified, so some ADH may have been present. The decay in the light reaction, presented in Fig. 2 of their publication, may be due to the conversion of decanal by ADH. They tried to prove the absence of aldehyde-attacking enzymes other than luciferase by a preincubation experiment in which they obtained more light after incubation with decanal and DPNH for 15 min than after incubation with these substances plus FMN for the same length of time. The result of this experiment, however, may be explained by the fact that oxidation of DPNH by DPNH-oxidase proceeds much faster in the presence of added FMN (*cf.* ref. 7). In other words, preincubation with FMN diminishes the DPNH concentration. Thus, this experiment does not prove that FMN is required for the utilization of aldehyde in the light reaction.

When considering the question of the conversion of aldehyde during the light reaction, it was established first (spectrophotometrically) that the presence of palmital does not detectably influence the rate of DPNH oxidation in an enzyme preparation containing FMN. It seems probable that the presence of aldehyde only causes a small part of FMNH to be oxidized *via* the light reaction instead of *via* the autoxidation pathway.

The light reaction, in the presence of excess DPNH and a limiting amount of palmital is constant for some time (Fig. 1a, b). The length of the horizontal part of the curve depends on DPNH supply. We examined whether the initial light reaction could be restored by addition of DPNH after a rather long reaction time (54 min). This proved to be possible in some experiments (Table II). Since some decrease of the enzyme activity (luciferase or DPNH-oxidase) may be expected during this relatively long incubation time, it does not seem surprising that not all experiments yielded this result. Therefore, we conclude that, as yet, there is no reason for assuming a conversion of long-chain aldehyde during its activity in the light reaction.

TABLE II

THE RESTORATION OF THE LIGHT REACTION WITH DPNH

Expt. No.	*Light reaction (counts/$^{7}/_{8}$ min)*		
	Initial	*After 50 min*	*After 54 min and addition of fresh DPNH*
1	608	120	634
2	282	79	259
3	376	128	303
4	236	36	254
5	55	9	51

Reaction mixture: 1 ml enzyme preparation, 1.2 mg DPNH in 0.5 ml phosphate buffer pH 7.0, 0.2 ml FMN 0.001 %, 0.02 ml palmital sol. 1 :20. DPNH + FMN were added before palmital. After 54 min about 0.5 mg DPNH was added. Measurement of the initial reaction at its highest rate (18 min after palmital addition), measurement of the restored reaction 4 min after DPNH addition. In Expt. 5 no palmital was added.

In the next experiments we always used palmital instead of decanal in order to avoid complications caused by a conversion of decanal independent of the light reaction.

B. The influence of iso-octane extraction of enzyme preparations on the light reaction

When no aldehydes were added all enzyme preparations showed a weak light reaction in the presence of DPNH and FMN. It was supposed, therefore, that the preparations contained some aldehyde (see section A). STREHLER AND CORMIER[1] found that a boiled extract of an acetonized bacterial preparation produces an increase in luminescence. Our boiled enzyme preparations, however, inhibited the light reaction if added to untreated preparations plus DPNH and FMN. We tried to extract the active substance by means of organic solvents. The evaporated extracts caused an inhibition of the light reaction. However, this did not prove the absence of long-chain aldehydes in the extracts since it appeared to be difficult to redissolve, after evaporation of the organic solvent, a small amount of palmital in buffer. We then tried to investigate whether the enzyme preparation had lost its active substance by the extraction. We therefore looked for a solvent that would not be likely to inactivate the enzyme preparation by protein denaturation, in order to be able to test the extracted preparation in the light reaction; iso-octane was used. Since the extraction of long-chain aldehyde, supposed to be present, was anticipated, it was expected that the extracted enzyme preparation would yield a decreased light reaction with DPNH and FMN. Contrary to expectation, however, the iso-octane-extracted enzyme system exhibited a clearly increased light reaction. Moreover, the relative increase of the light reaction in the presence of palmital is still higher (Table III).

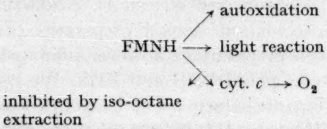
TABLE III

THE INFLUENCE OF ISO-OCTANE EXTRACTION ON THE LIGHT REACTION

Expt. No.	*Enzyme preparation*	*No palmital added*			*Palmital added*		
		Light reaction (counts/$^{7}/_{8}$ min)		*Ratio reactions extracted/un-extracted enzyme*	*Light reaction (counts/$^{7}/_{8}$ min)*		*Ratio reactions extracted/un-extracted enzyme*
		Enzyme not extracted	*Enzyme extracted with iso-octane*		*Enzyme not extracted*	*Enzyme extracted with iso-octane*	
1	1–12	1	3	—	107	184	1.7
2	12–12	18	33	1.8	1098	2519	2.3
3	12–12	20	94	4.7	972	7950	8.2
4	12–12	14	25	1.8	1062	3750	3.5
5	1–12	3	6	2.0	123	498	4.0
6	12–12	18	24	1.3	714	2632	3.7
7	1–12 12–12	7	14	2.0	208	509	2.4
8	12– 1	90	193	2.1	2320	3860	1.7
9	9– 2	41	53	1.3	1055	1865	1.8
10	9– 2 30– 1	33	172	5.2	1254	6700	5.3
11	30– 1	39	71	1.8	257	388	1.5
12	30– 1	40	111	2.8	257	587	2.3
13	16– 2	40	73	1.8	341	816	2.4
14	3– 4	10	13	1.3	44	124	2.8
15	3– 4	8	12	1.5	77	330	4.3

Reaction mixtures: 1 ml enzyme preparation, 0.5 mg DPNH in 0.5 ml phosphate buffer pH 7.0, 0.2 ml FMN 0.001 %, palmital solution 0.025 ml 1 :10 (Expts. 1–6), 0.01 ml 1 :10 (Expts. 7, 8, 9, 10, 15), 0.01 ml 1 :40 (Expts. 11, 12, 13), 0.025 ml 1 :40 (Expt. 14).

Iso-octane extraction of mitochondrial particles is known to cause a decrease of the cytochrome *c*-reductase activity[12], perhaps by removing some essential component in the electron-transfer system[13]. The effect of iso-octane extraction in our preparations might be due to the removal of such a component from a cytochrome *c*-reductase system competing with luciferase for FMNH:



TOTTER AND CORMIER[7] demonstrated that oxidized cytochrome *c* is an inhibitor of the light reaction. They ascribed this inhibition to competition between cytochrome *c* and luciferase for the H of FMNH. Assuming the presence of a cytochrome *c*-reductase system in our preparations, cytochrome *c* should cause no, or at least a decreased, inhibition of the light reaction in a preparation, extracted with iso-octane, if an essential component of the cytochrome *c*-reductase system had been removed. This proved not to be the case: addition of cytochrome *c* (about 0.9 mg/2 ml reaction mixture) caused an inhibition of the light reaction of 42 % in an unextracted preparation and of 44 % in a preparation extracted with iso-octane.

No absorption peak in the region between 4000 and 4500 Å could be detected in the concentrated iso-octane fraction, indicating the absence of measurable amounts of cytochrome components.

Furthermore, it was established that iso-octane extraction does not influence the rate of DPNH oxidation in the presence of FMN. The presence of palmital is without effect in this respect.

It was tested whether the effect of iso-octane extraction could be ascribed to some physical cause[14–16]. However, both with and without palmital, addition of some iso-octane to an enzyme preparation did not influence the light reaction.

Residues of evaporated iso-octane extracts appeared to inhibit the light reaction of enzyme preparations containing added palmital (Table IV). This result may indicate that iso-octane extracts an inhibitor from the enzyme preparations. This inhibitor does not influence DPNH oxidation.

TABLE IV

THE INFLUENCE OF EVAPORATED ISO-OCTANE EXTRACTS FROM ENZYME PREPARATIONS ON THE LIGHT REACTION OF NON-EXTRACTED ENZYME

Expt. No.	*Enzyme preparation*	*Light reaction (counts/$^{7}/_{8}$ min)*			
		No palmital added		*Palmital added*	
		No iso-octane extract	*Iso-octane extract*	*No iso-octane extract*	*Iso-octane extract*
1	1–12	1	1	107	58
2	12–12	18	18	1098	518
3	12–12	17*	17	1884*	664
4	1–12	3*	3	91*	54

* With iso-octane extract from buffer.

Reaction mixtures: iso-octane extract of 5 ml enzyme preparation taken up in 0.025 ml methanol and 0.25 ml phosphate buffer pH 7.0, 1 ml enzyme preparation, 0.5 mg DPNH in 0.25 ml buffer, 0.2 ml FMN 0.001 %, 0.025 ml palmital solution 1 : 10.

The question arose why iso-octane apparently does not, apart from this inhibitor, extract the long-chain aldehyde supposed to be present in the enzyme preparations. It appeared that iso-octane extracts only a part of the palmital added to an enzyme preparation, while extraction from buffer is much more complete (Table V). These results indicate that added palmital is adsorbed or bound to the enzyme preparation and thus prevented from being extracted. The same conclusion was drawn from earlier dialysis experiments, using decanal.

C. The influence of the sequence in which palmital, DPNH and FMN are added

In the earlier experiments carried out in order to determine the extractability of added palmital from enzyme preparations we often found that if a certain amount of palmital was added to an enzyme preparation prior to iso-octane extraction and the same amount was added to a similar preparation after iso-octane extraction (the control), more palmital (as estimated by means of the light reaction) seemed to be present afterwards in the first preparation than in the second one. This effect appeared to occur if, in the control, DPNH + FMN were added before the addition of palmital. We therefore investigated the influence of the sequence in which palmital,

TABLE V

THE EXTRACTABILITY OF PALMITAL WITH ISO-OCTANE FROM ENZYME PREPARATIONS AND FROM BUFFER

Expt. No.	*Palmital added (ml/ml enzyme)*	*Palmital (%) extracted from*	
		*Enzyme preparation**	*Phosphate buffer pH 7.0***
1a	0.008 (1:10)	4	
1b	0.008	13	
1c	0.008	33	
2a	0.008	38	
2b	0.008	6	
2c	0.008	0	
3	0.025		74
4a	0.01 (1:40)	33	
4b	0.01 (1:40)	24	
5	0.008 (1:10)		100
6	0.008 (1:10)		90
7a	0.025	26***	
7b	0.025		77***

* The light reaction with DPNH + FMN of (enzyme + palmital) extracted with iso-octane, compared with that of (enzyme extracted with iso-octane) + palmital.

In all controls DPNH + FMN were added after the palmital.

** The light reaction of enzyme + DPNH + FMN + {(palmital + buffer) extracted with iso-octaned}, compared with that of enzyme + DPNH + FMN + palmital + (buffer extracted with iso-octane).

*** Chemical estimation of aldehyde in the iso-octane fraction with *p*-nitrophenylhydrazine after WITTENBERG *et al.*[10].

TABLE VI

THE INFLUENCE OF THE SEQUENCE OF ADDITION OF PALMITAL AND DPNH + FMN ON THE LIGHT REACTION

Expt. No.	*Enzyme preparation*	*Sequence of addition of palmital (P) and DPNH + FMN (DF)*	*Light reaction (counts/$^7/_8$ min) in*		*Decrease of the light reaction (in %) on preincubation with DPNH + FMN*	
			Unextracted enzyme	*Enzyme extracted with iso-octane*	*Unextracted enzyme*	*Enzyme extracted with iso-octane*
1	30–1	P–DF	304	984	15	40
		DF–P	257	586		
2	16–2	P–DF	351	1185	3	31
		DF–P	342	816		
3	3–4	P–DF	205	781	6	37
		DF–P	193	491		
4	4–3	P–DF	127	498	27	43
		DF–P	93	286		

Reaction mixtures: 1 ml enzyme preparation, 0.5 ml DPNH in 0.5 ml phosphate buffer pH 7.0, 0.2 ml FMN 0.001 %, 0.01 ml palmital sol. (1:20 or 1:40). In every set of experiments the time during which DPNH + FMN were in contact with the enzyme was the same.

DPNH and FMN were added to the iso-octane-extracted preparation. There appeared to be a higher light production if palmital was added before DPNH and FMN, as compared with the light production in a preparation to which palmital was added after DPNH + FMN. The difference, although decreasing with time, was still clear

TABLE VII

THE INFLUENCE OF PREINCUBATION WITH DPNH + FMN, DPNH AND FMN ON THE LIGHT REACTION OF ENZYME PREPARATIONS EXTRACTED WITH ISO-OCTANE

		Decrease of the light reaction (%) of iso-octane-extracted enzyme upon preincubation with		
Expt. No.	*Enzyme preparation*	*DPNH + FMN (P–D–F versus D–F–P)*	*FMN (P–F–D versus F–P–D)*	*DPNH (P–D–F versus D–P–F)*
1	24–4	37	8	—
2	24–4	32	18	20
3	9–3	37	16	19

Reaction mixtures as described for Table VI. Symbols in parentheses indicate sequence of addition of the reactants. P: palmital, D: DPNH, F: FMN. In every set of experiments the time during which DPNH + FMN, DPNH and FMN were in contact with the enzyme was the same.

TABLE VIII

INFLUENCE OF FMN CONCENTRATION UPON THE DECREASE OF THE LIGHT REACTION OF ENZYME PREPARATIONS EXTRACTED WITH ISO-OCTANE ON PREINCUBATION WITH DPNH + FMN

Expt. No.	*Enzyme preparation*	*Concentration FMN added (%)*	*Light reaction (counts/$^1/_8$ min)*		*Decrease (%)*
			P + DF	*DF + P*	
1	9–3	0.01	226	81	64
		0.001	565	418	26
		0.0001	127	121	— 5
2	12–6	0.01	492	298	40
		0.001	1320	865	35
		0.0001	310	264	15

Reaction mixture as described for Table VI. P: Palmital. DF: DPNH + FMN. Sequence of symbols indicates sequence of addition of the reactants.

TABLE IX

INFLUENCE OF PALMITAL CONCENTRATION UPON THE DECREASE OF THE LIGHT REACTION IN ENZYME PREPARATIONS EXTRACTED WITH ISO-OCTANE ON PREINCUBATION WITH DPNH + FMN

Expt. No.	*Enzyme preparation*	*Concentration palmital added (ml)*	*Light reaction (counts/$^1/_8$ min)*		*Decrease (%)*
			P + DF	*DF + P*	
1	29–5	0.02	2114	1924	9
		0.02 (1 : 5)	353	315	11
		0.02 (1 :20)	108	63	42
		0.02 (1 :80)	34	18	47
2	12–6	0.02	1581	1486	6
		0.02 (1 : 5)	602	446	26
		0.02 (1 :20)	272	173	36
		0.02 (1 :80)	102	63	38

Reaction mixture as described for Table VI. P: Palmital, DF: DPNH + FMN. Sequence of symbols indicates sequence of addition of the reactants. The value of the light reaction without added palmital has been subtracted.

after 15–20 minutes. The effect was likewise present in non-extracted preparations, though to a much smaller extent (Table VI). The time during which DPNH + FMN were in contact with the enzyme (varying from 7 sec to 10 min) did not influence the result. Preincubation with either DPNH or FMN caused a similar effect. However, the difference was smaller than after preincubation with DPNH + FMN (Table VII).

In former experiments[17] we never observed this influence of the sequence of addition of the various components to *Ph. phosphoreum* preparations. Rather was the reverse effect sometimes observed (*cf.* Fig. 1 in ref.[17]). This may now be explained by the facts that: (1) the effect is most clear in preparations extracted with iso-octane, (2) when using decanal, as was done in our previous experiments, the effect is counteracted by the conversion of decanal, independent of the light reaction.

In order to obtain more insight into the cause of the phenomenon described above we studied the influence of the concentration of FMN and palmital. The difference (in percentage) between the light reactions produced by adding first palmital as compared with that obtained by adding first DPNH + FMN appeared to increase with increasing amounts of FMN, while a decrease was observed with increasing palmital concentrations (Tables VIII and IX).

DISCUSSION

Before trying to suggest an explanation for the phenomena described B, the experimental results may be briefly summarized as follows:

(1) Iso-octane extraction of enzyme preparations increases the light reaction, probably by removal of an inhibitor. It has no detectable influence on the DPNH-oxidase activity. As a rule the iso-octane effect is more pronounced when palmital is added than when it is not.

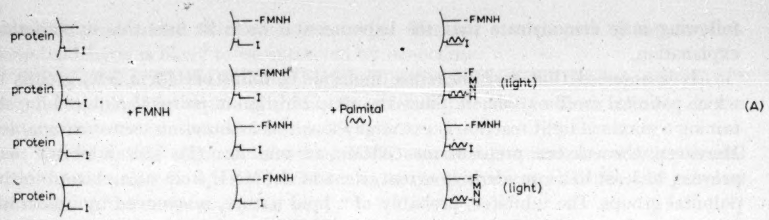
(2) In iso-octane-extracted enzyme preparations the light reaction depends on the sequence in which palmital and DPNH + FMN are added. If palmital is added first the light reaction is for a considerable period higher than if palmital is added last. This effect is observed to a lesser extent in unextracted enzyme preparations.

(3) Palmital added to an enzyme preparation is only slightly removed by iso-octane extraction.

(4) There is no evidence for a chemical change of aldehyde in the light reaction.

On the basis of previous experiments we developed a scheme for the luciferase molecule in which two different sites were present, one for binding FMN(H) and one for binding long-chain aldehydes. The site of FMNH might be occupied by aldehyde[17]. This scheme, however, fails to explain all the experimental facts mentioned above, especially the long-lasting "addition sequence effect" mentioned under (2). In the literature we found one instance of an effect comparable with that in question: in testing the influence of the order of addition of the components on the reconstruction of carboxylase activity, GREEN *et al.*[18] observed that the greatest activity was found if magnesium was added to the protein prior to diphospho-thiamine. According to LEHNINGER[19] this suggests that Mg^{++} serves as the binding agent between protein and diphospho-thiamine.

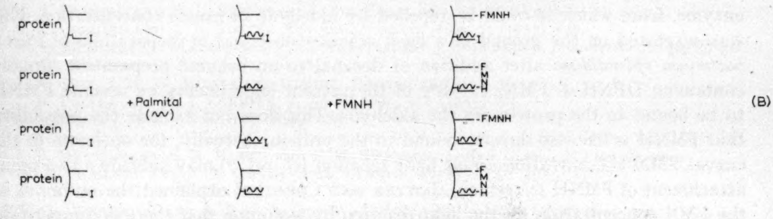
If we apply the suggestion of LEHNINGER to our enzyme system, *i.e.* if we consider the long-chain aldehyde as the binding group between the enzyme and FMN(H), the


protein
I
+FMNH
-FMNH
+ Palmital (~~)
F M N H
(light)
(A)

Unextracted enzyme molecules.

FMNH takes its sites at the enzyme. It cannot block the sites screened by I.

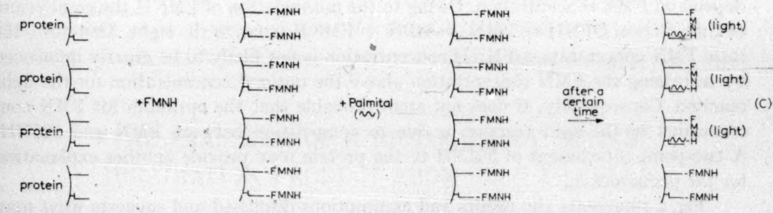
Palmital decreases the affinity of I to the enzyme, the light reaction can take place at the uninhibited enzyme.


protein
I
+ Palmital (~~)
+FMNH
-FMNH
M N H
(B)

Unextracted enzyme molecules.

Palmital occupies the enzyme, the affinity of I to the enzyme is decreased.

FMNH occupies its sites, the light reaction occurs at the uninhibited enzyme.

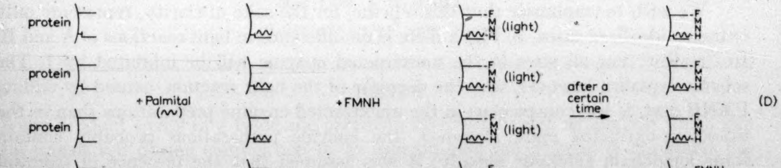

protein
+FMNH
-FMNH
+ Palmital (~~)
after a certain time
F M N H
(light)
(C)

Iso-octane extracted enzyme molecules.

FMNH blocks the palmital sites.

Palmital cannot immediately occupy its sites.

The equilibrium is established.


protein
+ Palmital (~~)
+FMNH
F M N H
(light)
after a certain time
-FMNH
(D)

Iso-octane extracted enzyme molecules.

Palmital occupies its sites.

The light reaction takes place uninhibited.

The equilibrium is established.

Fig. 2. Explanation see text.

following may demonstrate that the experimental facts fit into this hypothetical explanation.

Is is assumed that each luciferase molecule contains one (or a few) groups to which palmital can be attached. (Since the concentration of palmital required for obtaining a maximal light reaction is extremely low, this assumption seems reasonable.) Moreover, the enzyme preparations contain an inhibitor (I). This inhibitor may prevent, at least to a considerable extent, the added FMNH from being bound to the palmital groups. The inhibitor, probably of a lipid nature, is removed by iso-octane extraction. It is assumed, furthermore, that FMN(H) can block the groups of the enzyme molecules that are to bind palmital. Such a blocking may be likewise inhibited by the inhibitor. Besides, we have to assume that the affinity of the inhibitor to the enzyme decreases if the enzyme is occupied with palmital.

In our previous article[17] we assumed the presence of a site for FMNH at the enzyme, from which it could be repelled by aldehyde in excess concentration. This was suggested on the ground of a flash reaction observed in preparations of *Photobacterium splendidum* after addition of decanal to an enzyme preparation already containing DPNH + FMN. In view of the present experiments we assume FMNH to be bound to the protein *via* the aldehyde. This does not exclude the possibility that FMNH is likewise directly bound to the protein. Actually, the optimum in the curve: FMN concentration *versus* light reaction (*cf.* ref. 7) may indicate a two-point attachment of FMNH (*cf.* ref. 20). TOTTER AND CORMIER[7] explained the optimum in the FMN concentration for the light reaction by assuming that there is competition between FMN and FMNH for a site at the luciferase molecule. However, the FMN concentration is still limiting for the DPNH-oxidase reaction in the same enzyme preparation. Therefore, the velocity of DPNH oxidation (and FMN reduction) will depend on FMN concentration. Owing to the autoxidation of FMNH the equilibrium of the reaction DPNH + FMN $\rightarrow$ DPN + FMNH is far to the right. Therefore, the ratio FMN concentration/FMNH concentration is not likely to be greatly influenced by increasing the FMN concentration above the optimal concentration for the light reaction. Consequently, it does not seem probable that the optimum for FMN concentration in the light reaction is due to competition between FMN and FMNH. A two-point attachment of FMNH to the protein may provide another explanation for the phenomenon.

Fig. 2 represents the results and assumptions discussed and suggests what may happen if palmital and FMNH are added to the enzyme, which has or has not been extracted with iso-octane.

We wish to emphasize that this scheme, for the sake of clarity, represents only extreme, idealized cases. In Fig. 2 there is no difference in light reactions of A and B. In "reality" not all sites in the unextracted enzyme will be inhibited by I. The scheme explains, however, why the decrease of the light reaction, caused by adding FMNH first, is less conspicuous in the unextracted enzyme preparations than in the iso-octane-extracted ones. Moreover, the enzyme preparations probably contain some long-chain aldehyde already; it was assumed that the presence of palmital decreases the affinity of the inhibitor to the enzyme. We may suppose, therefore, that the percentage of the aldehyde-occupied sites inhibited by I is smaller than the percentage of aldehyde-free sites inhibited by I. This may explain why the experimental results seemed to indicate that the effect of iso-octane extraction is less when palmital is not added than when it is added. Besides, a small part of the enzyme-bound aldehyde is likely to be extracted by iso-octane.

The fact that FMN and DPNH are both active in the "addition sequence effect" may be explained by assuming that both molecules are able to block the sites on the enzyme that are "intended" for the aldehyde.

The increasing effect at increasing FMN concentration and decreasing palmital concentration may be understood in terms of competition of FMN(H) and palmital for a site on the enzyme.

It may be remarked that the presented scheme should be considered as a working hypothesis. So far, it offers an explanation of the experimental results.

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THE TRANSFER OF A BIOLOGICALLY ACTIVE IRRADIATION PRODUCT FROM CELL TO CELL*

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SUMMARY

Some product exists in a lysogenic organism after ultraviolet irradiation which can be transferred during conjugation to an unirradiated cell wherein it duplicates the effects of direct irradiation. The product is stable in the cold, unstable at 37° and is unstable to illumination by visible light. The nature of the irradiated product is unknown but it is apparently not the prophage.

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* A preliminary report has been published[1].

INTRODUCTION

The mechanism of the conversion of incident ultraviolet energy into biological consequences in microorganisms is obscure. Several investigators have postulated that the lethal effect of the irradiation of microorganisms is not direct but that it is mediated by the formation of some toxic products under whose influence the subsequent physiological processes ensue[2,3]. However, the existence of such products with which the irradiated organism is presumably emburdened has not been demonstrated.

From studies of the induction of lysogenic microorganisms by ultraviolet irradiation[4] we also arrived at the tentative conclusion that there are mediating agents between the radiant energy and the complex of biological changes which it precipitates.

We report here evidence for the existence of some relatively stable product of irradiation in microorganisms which when transferred to an unirradiated lysogenic cell duplicates the effects of direct irradiation. So far we have been able to transfer the products of irradiation from cell to cell only during conjugation.

The principle of these experiments is as follows. Auxotrophic, lysogenic *E. coli* K_{12} cells with an F^+ or Hfr sexual orientation are deprived of an essential nutrient. Then, after irradiation they are permitted to conjugate with inducible lysogenic F^- cells and the consequent phage formation is assayed. Since the irradiated auxotrophs were shown to be unable to produce phage under these conditions the yield of phage must stem from the unirradiated F^- cells. Confirmation of this view was obtained by irradiating non-lysogenic F^+ cells and obtaining phage from lysogenic F^- cells after permitting these two cultures to conjugate. In this instance, the phage yield must unequivocally stem from the unirradiated F^- cells since the irradiated F^+ cells carry no prophage.

The phenomenon, which we shall call cross-induction offers some advantages for the study of the mechanism of induction. Since the cell which received the irradiation and the one in which the phage develop are different, the effect of various stresses on each event can be separately studied. Moreover a study of this phenomenon may contribute towards our understanding of radiobiological mechanisms.

The phenomenon is not restricted to the *E. coli* K_{12} system. Dr. BRUCE HOLLOWAY[5] of the University of Melbourne has repeated these experiments on a lysogenic *Pseudomonas pyocyanea* in which sexual recombination is also possible and confirmed the findings we reported with *E. coli* K_{12}.

EXPERIMENTAL

Microorganisms

The microorganisms used are enumerated here with their pertinent attributes and, for brevity, a code letter is assigned to each:

A: *E. coli* K_{12} W6 F^+ M^- Lp^+ (a methionine-requiring, prophage-carrying male).

B: *E. coli* K_{12} W 1177 F^- Thr^- Leu^- B_1^- Lp^+ (a leucine-, threonine- and thiamine-requiring, prophage-carrying female).

C: *E. coli* K_{12} P4x Hfr M^- Lp^+ (a methionine-requiring male with high efficiency of genetic transfer; contains prophage) isolated and kindly provided by Dr. FRANÇOIS JACOB.

D: *E. coli* K_{12} W 1817 F^+ Thr^- Leu^- B_1^- Lp^+ (the male counterpart of B).

E: *E. coli* K_{12} W 1485 F^+ Lp^s (a male oriented organism lacking a lysogenic prophage but sensitive to the phage).

All these organisms, except C, were isolated and were kindly provided by Dr. JOSHUA LEDERBERG.

As the indicator strain for the assay of *lambda* the organism isolated by Dr. R. K. APPLEYARD, *E. coli* K_{12} C-600 was used.

Typical experiment

E. coli K_{12} W6 was grown from a small inoculum to logarithmic growth phase in a chemically defined medium[6] supplemented with 30 μg/ml of DL-methionine. When the culture reached a density of about $5 \cdot 10^8$ cells/ml, it was centrifuged, and the cells were washed once with the growth medium lacking methionine. The organisms were resuspended to a cellular concentration of $1 \cdot 10^8$/ml in the medium lacking methionine and were irradiated at 1 m distance with a 15 W GE germicidal lamp for varying periods. Immediately after the irradiation 5.0 ml of the culture was mixed with a washed culture of 5.0 ml of *E. coli* K_{12} W1177 in a medium supplemented with 250 μg DL-leucine, 500 μg DL-threonine and 1 μg thiamin per ml. Since this medium lacked methionine it could not support phage formation in the methionine-requiring organism with the male sexual orientation. The mixture of organisms was incubated for 140 min in the dark. The culture was then centrifuged for 20 min at 5000 rev./min to sediment the cells and free λ phage was assayed in the supernatant fluid by the usual technique[7]. To determine the background level of phage contributed by the F^+ auxotroph, part of the irradiated culture was separately incubated without methionine for 140 min in the dark and free phage was assayed as described above. Aliquots of the F^- culture were also incubated separately, as well as with unirradiated F^+ cultures, to determine the free phage yielded under these conditions.

An alternative procedure for the conjugation was found to produce a higher free phage titre. The mixed culture was diluted 10^4-fold after allowing conjugation to proceed for brief periods and was then incubated for a total of 140 min. This method probably minimizes the loss of free phage which occurs by the absorption of it into some of the cells.

E. coli K_{12} W1485 which does not carry a prophage and therefore cannot give rise to bacteriophage was not deprived of any nutrient prior to irradiation. A culture in logarithmic growth phase with a cellular concentration of $1 \cdot 10^8$/ml was irradiated and 5.0 ml of it was admixed with 5.0 ml of *E. coli* K_{12} W 1177, as above.

For the photorestoration effects the cultures were immersed in an ice-water bath and the light from a mercury vapor lamp Hanovia H250-A5 (max 360–370 mμ) was focused on them. The unilluminated controls for these experiments were cultures in a masked tube immersed in the ice bath where the illumination was taking place.

RESULTS

In Table I, Expt. 1, the data for a characteristic experiment involving the mating of an irradiated F^+ with an unirradiated F^- organism are presented. When the irradiated methionine-requiring auxotroph is incubated alone without methionine, the phage yield is less than 10^5/ml. When it is mixed with the receptor F^- organism, in a medium adequate for the growth of the latter, the phage yield is $3 \cdot 10^7$/ml. The receptor organism alone during the same period produces $2 \cdot 10^6$ phage per ml. Therefore the yield of phage resulting from the admixture of the two cultures is at least $2.8 \cdot 10^7$.

If the conjugating culture is diluted as in Expt. 1a, the yield of free phage increases close to 100-fold over the background to $1.6 \cdot 10^8$. The burst size of these organisms in synthetic medium is approx. 15. Therefore, the number of cells which produced phage in the diluted sample can be calculated to be $1 \cdot 10^7$. Since the number of irradiated F^+ cells in the conjugating culture was known to be $5 \cdot 10^7$, the effective transfer of induction under these conditions was about 20 %.

TABLE I

Experiment	*Description of experiment*	*Irradiated organism No/ml·10⁻⁷*	*Irradiated sec*	*Background phage from irradiated organism*	*Unirradiated organism No/ml·10⁻⁷*	*Background phage in 140 min*	*Total phage in 140 min*
1	Standard F^+ to F^-	A 5.0	400	10^5	B 8.0	$2 \cdot 10^6$	$3 \cdot 10^7$
1a	Same as 1 but diluted in 20′ 10^4-fold	A 5.0	400	10^5	B 8.0	$2 \cdot 10^6$	$1.1 \cdot 10^8$
2	Hfr to F^-	C 6.0	400	10^5	B 9.0	$1 \cdot 10^7$	$3 \cdot 10^7$
2a	Same as 2 but diluted in 8′ 10^4-fold	C 6.0	400	10^5	B 9.0	$1 \cdot 10^7$	$9.6 \cdot 10^7$

In Expts. 2 and 2a, an auxotrophic Hfr strain replaced the F^+ organism of Expts. 1 and 1a. The cross induction with the Hfr organism as measured by phage yield was consistently smaller than with the F^+ organisms. The reason for this difference is obscure at the present time.

In Table II, some of the experiments designed to probe the conditions necessary for cross-induction and some of the properties of the irradiation product are summarized. In Expts. 1 and 2, Table II, the stability of the irradiation product at 5° and 37° was studied.

TABLE II

Experiment	*Description of experiment*	*Irradiated organism No/ml·10⁻⁷*	*Irradiation sec*	*Unirradiated organism No/ml·10⁻⁷*	*Background phage in 140 min*	*Total phage in 140 min*
1	F^+ to F^-	A 7	180	B 7	$4.5 \cdot 10^6$	$3.6 \cdot 10^7$
1a	The same F^+ culture after 22 h at 5°	A 7	180	B 6	$5 \cdot 10^6$	$3.7 \cdot 10^7$
2	F^+ to F^-	A 7	180	B 12	$5.5 \cdot 10^6$	$2.9 \cdot 10^8$
2a	The same F^+ culture after 1 h at 37°	A 7	180	B 12	$5.5 \cdot 10^6$	$8.9 \cdot 10^7$
3	F^+ to F^+	A 5.0	180	D 5.0	$9 \cdot 10^5$	$1.2 \cdot 10^6$
4	F^- to F^+	B 5.0	180	A 5.0	$2 \cdot 10^6$	$4 \cdot 10^6$
5	$F^+\lambda^-$ to $F^-\lambda^+$	E 7.0	400	B 7.0	$3.7 \cdot 10^6$	$7.8 \cdot 10^7$

In these experiments, the irradiated F$^+$ cultures were kept at 5° and at 37° without methionine for the periods indicated, and were then allowed to conjugate with a fresh culture of the F$^-$ organisms in logarithmic growth phase. At 5° the ability of the F$^+$ organisms to transfer induction is unimpaired even 22 h after the irradiation, however, at 37° there is a 70 % reduction in one hour.

That cross-induction does not occur extensively between organisms of similar sexual orientation is indicated by the findings in Expt. 3, Table II. Here the irradiated donor was admixed with an auxotroph of similar sexual orientation, *E. coli* K_{12} W 1817 F$^+$. The yield of phage was only slightly above the background, indicating only a slight cross-induction.

The negative findings in the homosexual crossing also serve to eliminate mechanisms other than cross-induction on the basis of the observed phenomenon. It eliminates the possibility that the increased phage formation is due to the cross-feeding of methionine to the irradiated donor. As further evidence of this, we have incubated irradiated *E. coli* K_{12} W6 in sterile culture fluids in which the recipient cells, *E. coli* K_{12} W 1177 had grown to late logarithmic growth phase. No enhancement of phage formation could be detected indicating that there had been no leakage of methionine into the medium by the auxotroph which is capable of synthesizing this amino acid.

The negative findings in the homosexual crossing also rule out the possibility that the enhanced phage formation is due to the irradiation of the medium itself.

That cross-induction is unidirectional, going only from F$^+$ to F$^-$ organisms is indicated by Expt. 4, Table II. Here *E. coli* K_{12} W 1177F$^-$ was irradiated in a medium lacking threonine, leucine, and thiamin and was admixed with *E. coli* K_{12} W6 F$^+$ in a complete medium for the latter. No significant excess phage formation was observed.

JACOB AND WOLLMAN[8] have discovered that if a lysogenic F$^+$ organism is permitted to conjugate with a non-lysogenic F$^-$ organism, phage production ensues, a phenomenon to which they refer as zygotic induction. Therefore, it was essential to determine that in Expt. 1 we did not encounter some variant of zygotic induction. We found that irradiation of the F$^+$ population is an obligatory prerequisite to cross-induction for if the F$^+$ and F$^-$ organisms were admixed without previous irradiation and were incubated, no increase in phage titre over the background was obtained.

To explore the nature of the irradiated product which effects cross-induction, its stability on exposure to illumination with visible light under various conditions was investigated.

In Expt. 1, Table III, the F$^+$ culture which had been irradiated with ultraviolet was exposed for 1 h to visible light at 0°.

A 70 % reduction in cross-induction was observed compared to the control which was an aliquot of the u.v.-irradiated culture which had been kept in the dark at 0° for 1 h.

It has been suggested by DULBECCO[9] that the effect of visible light on induced cultures of lysogenic organisms may not be solely photo-restoration but there may be inhibition of phage development by the visible light. The experiment cited above would seem to indicate that photo-restoration cannot be completely excluded as a mechanism in the reversal of induction by visible light in a lysogenic organism. Under the conditions of our experiment, the cell in which the phage develops was

TABLE III

Experiment	*Description of experiment*	*Irradiated organism* $No/ml\cdot10^{-7}$	*Irradiation sec*	*Unirradiated organism* $No/ml\cdot10^{-7}$	*Background phage in 140 min*	*Total phage in 140 min*
1	F^+ to F^-	A 5	400	B 6	$2.6\cdot10^6$	$2.6\cdot10^7$
1a	F^+ illuminated for 1 h after u.v.	A 5	400	B 6	$2.6\cdot10^6$	$7.7\cdot10^6$
2	F^+ to F^-	A 3	400	B 10	$3.9\cdot10^6$	$1\cdot10^8$
2a	F^+ illuminated for 1 h prior to u.v.	A 3	400	B 10	$3.9\cdot10^6$	$1.1\cdot10^7$
3	F^+ to F^-	A 5	400	B 10	$4.5\cdot10^6$	$2.4\cdot10^7$
3a	F^- illuminated for 1 h prior to conjugation	A 5	400	B 10	$4.5\cdot10^6$	$5\cdot10^6$

not exposed to illumination by visible light. On the other hand, there is a possibility that there is a transfer of the effects of illumination as well as of products of the u.v. irradiation during conjugation. The following experiment was designed to detect whether any illumination products can be transferred during conjugation. A culture of F^+ organisms was illuminated with visible light and was permitted to conjugate with a culture of F^- lysogenic organism which had been induced by u.v. irradiation. There was no diminution of phage yield compared with a control in which the induced F^- cultures were permitted to conjugate with an un-illuminated aliquot of the F^+ culture. Therefore, the products of illumination, unlike the products of u.v. irradiation, do not appear to be transferred during conjugation.

In Expt. 2, Table III, the F^+ culture was exposed to visible light for one hour prior to exposure to the inducing dose of u.v. irradiation. A 90 % reduction in cross-induction was found by such treatment. This finding parallels those obtained from experiments where the effects of prior illumination were studied on the lethal effects of u.v. irradiation[10].

In Expt. 3, Table III, the F^- recipient culture was exposed to illumination by visible light for 1 h prior to cross-induction with an u.v.-irradiated F^+ culture. The pre-illumination of the F^- culture eliminated any measurable cross-induction.

DISCUSSION

The experiments described here point to the existence of some component of a lysogenic cell which is the target of u.v.-irradiation and which can be transferred during conjugation from the irradiated male to the unirradiated female where it simulates the effect of direct irradiation by producing induction of the prophage.

The phenomenon is constantly reproducible. In over a hundred experiments there has been no loss of it. Quantitatively, however, there is considerable variation in the phage yield. This is not surprising in view of the complexity of these experiments: the phase of the culture of the F^+ organisms may affect both their aptitude[11] and the efficiency of the transfer; the phase of the culture of the F^- organisms may affect the efficiency of the receipt and of phage development.

The irradiated product is stable in the cold, unstable at 37° and is unstable when illuminated by visible light. The nature of the irradiated component of the cell which effects cross-induction is obscure at the present time. One locus, the prophage, however, is quite certainly eliminated. There are two lines of evidence which tend to rule out the transfer of an irradiated prophage as the mechanism of cross-induction.

We have been able to effect cross-induction by conjugating an irradiated F^+ organism which carries no prophage (see Expt. 5, Table II). Dr. BRUCE HOLLOWAY[5] was also successful in effecting cross-induction with a non-lysogenic F^+ parent of *Pseudomonas pyocyanea*. Moreover, he identified the progeny from a doubly lysogenic F^- parent when it was subjected to cross-induction and found that both of the prophages of the female parent were induced.

The cross-inducing material may be part of the genetic material other than the prophage or it may be some cytoplasmic component. A study of its nature is under way.

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MULTI-MEMBRANE FILTRATION

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SUMMARY

The theory for a multiple stage filtration, under conditions in which solute transport by diffusion is negligible compared with that by filtration flow, is given. Three examples of protein filtration are analyzed by an efficient statistical method. In this method a Gaussian-type iterative scheme is used for finding the maximum likelihood estimates of the unknown physical constants of the system.

INTRODUCTION

Multiple stage filtration through membranes has so far not been used for separating substances to which the membranes are considerably permeable. It is therefore of interest to consider the effectiveness for this purpose of a multi-membrane arrangement such as that shown in Fig. 1.

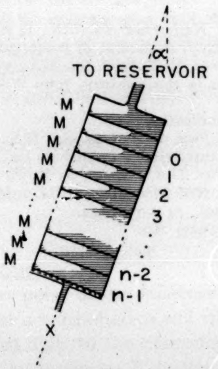

Fig. 1. Schematic diagram of the Multi-Membrane Filtration Apparatus. M = membrane; the compartments are numbered as indicated on the right; the fluid enters at the top.

A cylinder is divided by $(n + 1)$ membranes into n compartments, numbered 0 to $(n - 1)$. In compartment 0 is the solution containing the solute, in all other compartments is solvent. In every compartment a space is left empty so that, when the device is made to revolve along the surface of a cone of angle α, but without rotation about its own axis X, the air bubbles act as stirrers. When solvent from the reservoir at the top is forced downwards at constant pressure, liquid enters successively in all compartments until a stationary state is reached in which the volume element, dv, entering each compartment is equal to that leaving it, ($-$ dv). The following simplifying assumptions are made: (a) The stationary state is reached at time zero when all the solute is still contained in compartment 0. (b) All volumes of liquid in the compartments are equal to v_0. (c) The membranes are equally retentive for any individual solute species independently of concentration so that the ratio of concentrations on the two surfaces of any one membrane $c_{\text{bottom}}/c_{\text{top}} = f$ is constant. (d) Mixing of the fluids is immediate. (e) Solute is not adsorbed in the membrane. (f) At the bottom of the device solution is removed in differential portions of size dv.

It can then be shown by solving successively equations (1)

$$\begin{aligned}
\mathrm{d}m_0 &= -f\frac{m_0}{v_0}\mathrm{d}v \\
\mathrm{d}m_1 &= +f\frac{m_0}{v_0}\mathrm{d}v - f\frac{m_1}{v_0}\mathrm{d}v \\
\mathrm{d}m_2 &= +f\frac{m_1}{v_0}\mathrm{d}v - f\frac{m_2}{v_0}\mathrm{d}v \\
&\vdots \\
\mathrm{d}m_k &= +f\frac{m_{k-1}}{v_0}\mathrm{d}v - f\frac{m_k}{v_0}\mathrm{d}v \\
&\vdots \\
\mathrm{d}m_{n-1} &= +f\frac{m_{n-2}}{v_0}\mathrm{d}v - f\frac{m_{n-1}}{v_0}\mathrm{d}v \\
\mathrm{d}m_n &= +f\frac{m_{n-1}}{v_0}\mathrm{d}v \text{ (outflow)},
\end{aligned} \tag{1}$$

where m_k is the amount of solute in compartment k as a function of the volume v passed through, that

$$m_k = \frac{m_A}{k!}\left(\frac{f}{v_0}\right)^k v^k \exp\left(-fv/v_0\right) \tag{2}$$

where m_A is the total initial amount of solute.

The concentration c_n at the outflow is given by

$$\begin{aligned}
c_n = c_{n-1}f = m_{n-1}\frac{f}{v_0} &= \frac{m_A}{(n-1)!}\left(\frac{f}{v_0}\right)^n v^{n-1} \exp\left(-fv/v_0\right) \\
&= \frac{c_A}{(n-1)!} f^n \left(\frac{v}{v_0}\right)^{n-1} \exp\left(-fv/v_0\right),
\end{aligned} \tag{3}$$

where c_A is the initial concentration in compartment 0.

By equating $(\mathrm{d}c_n/\mathrm{d}v)$ to zero one obtains $v_{\max}$, the volume passed at which c_n is at a maximum:

$$\frac{\mathrm{d}c_n}{\mathrm{d}v} = \frac{m_A}{(n-1)!}\left(\frac{f}{v_0}\right)^n\left[(n-1)v^{n-2} - \frac{f}{v_0}v^{n-1}\right]\exp\left(-fv/v_0\right) = 0$$

$$\frac{f}{v_0}v_{\max}^{n-1} = (n-1)\,v_{\max}^{n-2}$$

$$\frac{v_{\max}}{v_0} = \frac{n-1}{f} \tag{4}$$

TABLE

VALUES OF c_n/c_A

v/v_0	1	2	3	4	5	6	7	8
$f = 1$	0.0005	0.0120	0.0504	0.104	0.146	0.161	0.149	0.122
$f = 0.5$	—	0.00025	—	0.0060	—	0.025	—	0.052
Ratio	—	$\frac{48}{1}$	—	$\frac{17.3}{1}$	—	$\frac{6.4}{1}$	—	$\frac{2.3}{1}$

Inserting (4) into (3), we have

$$\frac{(c_n)_{\max}}{c_A} = \frac{1}{(n-1)!} f^n \frac{(n-1)^{n-1}}{f^{n-1}} \exp[-(n-1)] = \frac{(n-1)^{n-2}}{(n-2)!} f \exp[-(n-1)] \tag{5}$$

and

$$\frac{(c_n)_{\max}}{(c_n)_{\max,\, f=1}} = f, \quad \frac{v_{\max}}{v_{\max,\, f=1}} = \frac{1}{f} \tag{6}$$

Equations (6) express the fact that the relative maximum concentration in the filtrate is inversely proportional to the volume filtered at which the maximum concentration occurs.

The first two rows of Table I show c_n/c_A as a function of v/v_0 for $f = 1$ and $f = 0.5$ calculated to apply in the case of a 7-compartment device. The relative concentration at $v = rv_{\max}$ is given by (4) and (3):

$$\begin{aligned} \frac{c_n}{c_A} &= \frac{1}{(n-1)!} f^n r^{n-1} \left(\frac{v_{\max}}{v_0}\right)^{n-1} \exp(-frv_{\max}/v_0) \\ &= \frac{1}{(n-1)!} f^n r^{n-1} \frac{(n-1)^{n-1}}{f^{n-1}} \exp[-fr(n-1)/f] \\ &= \frac{(n-1)^{n-2}}{(n-2)!} f r^{n-1} \exp[-r(n-1)] \end{aligned} \tag{7}$$

Assuming in the solution two solutes 1 and 2 do not interact and are retained to the extent given by f_1 and $f_2 = f_1/r$, one may calculate their proportions when each appears at maximum concentration in the filtrate.

By (6)

$$rv_{\max,\, f_1=f} = v_{\max,\, f_2=f/r}$$

at which volume, by (5)

$$\frac{(c_n)_{\max,\,2}}{(c_A)_2} = \frac{(n-1)^{n-2}}{(n-2)!} \frac{f}{r} \exp[-(n-1)] \tag{8}$$

Assuming further, for simplicity's sake, that $(c_A) = (c_A)_2$, then the ratio of concentrations of 2 and 1 at the concentration maximum of 2 is given by the ratio of (8) to (7)

$$\frac{(c_n)_{\max,\,2}}{(c_n)_1} = r^{-n} \exp[(r-1)(n-1)],$$

and at the concentration maximum of 1 by the ratio of (7) to (5),

$$\frac{(c_n)_2}{(c_n)_{\max,\,1}} = r^{n-2} \exp[-(r-1)(n-1)].$$

I

FOR FILTRATE

10	12	14	16	20	24	28	32
0.0625	0.0255	0.0087	0.0026	0.0002	—	—	—
0.073	0.080	0.075	0.061	0.031	0.013	0.0044	0.0013
$\frac{1}{1.2}$	$\frac{1}{3.1}$	$\frac{1}{8.6}$	$\frac{1}{20}$	$\frac{1}{150}$	—	—	—

These expressions may be taken as a measure of the degree of separation of two components.

For example, with a 7-component apparatus and for $r = 2$, which corresponds to $f_2 = f_1/2$,

$$\frac{(c_n)_{\max,2}}{(c_n)_1} = 2^{-7} \exp(6) = 3.15 \text{ and } \frac{(c_n)_2}{(c_n)_{\max,1}} = 2^5 \exp(-6) = 0.08.$$

In order that the first of the two values be greater than unity

$$n > \frac{r - 1}{r - 1 - \ln r} \tag{9}$$

Thus for $f_2 = 0.9 f_1$ or $r = 1.11$, n has to be at least 20.

A practicable procedure might be to continue the experiment until $v = rv_{\max,1}$, with r in the neighborhood of 2, and then to collect the contents of the compartments. The relative concentrations of the two components in any compartment k is given from (2) by

$$\frac{m_{k,2}}{m_{k,1}} = \left(\frac{f_2}{f_1}\right)^k \exp\left[-r\left(\frac{f_2}{f_1} - 1\right)(n - 1)\right]. \tag{10}$$

With a 7-compartment apparatus and for $r = 2$ the relative concentrations of the two components in the compartments are, starting at the top of the apparatus: 403, 202, 101, 50.4, 25.2, 12.6, 6.3. The portions of component 2 left in the compartments are given by (2)

$$\frac{m_{k.2}}{m_{A.2}} = \frac{r^k(n - 1)^k}{k!} \left(\frac{f_2}{f_1}\right)^k \exp\left[-\frac{f_2}{f_1} r(n - 1)\right]. \tag{11}$$

For our example these are: 0.0025, 0.015, 0.045, 0.090, 0.134, 0.161; total = 44.8 % of component 2 originally present. At $v = 2v_{\max,1}$ a total of 4.76 % of component 1 is left within the compartments.

Experiments were made with a 5-compartment apparatus*. The membranes used were selected from a batch of the "very dense" Membrane Filters Type A (average pore size $< 0.1\ \mu$) made by Schleicher & Schuell, Keene, N.H., so as to have flow times between 5.5 and 6.8 min/ml for 6.6 cm^2 exposed area at 22 ($\pm$ 1)° and 2 lbs/in^2. In order to eliminate irreversible adsorption of protein in the experiments the dry membranes were placed in 1 + 4 diluted serum subsequently removed by filtering 20 ml buffer solution through each membrane separately. The same set was used in all experiments, with thorough backwashing in between experiments until the filtrate gave an absorbancy of 0.005 or less. The volume of each compartment was 1 ml, half of which was taken up by liquid. A buffer solution, 0.01 M in each Na_2HPO_4 and NaH_2PO_4 and 0.1 or 1.0 M in KCl, served as solvent. Bovine plasma albumin (BPA) was obtained from Armour Company, Chicago, and used as a 2-% solution. Edestin, 2× crystalline, was supplied by National Biochemical Corporation, Cleveland. A 2-% suspension in buffer solution containing 1 M KCl was warmed to 45° and centrifuged at 22° for 1 h. The supernate was taken off, cooled to 17° and centrifuged at 22° to remove precipitate which had formed on cooling. The supernate which was now completely clear was used for the experiment. Serum from a healthy blood donor was diluted with two parts of buffer solution before use. The concentration of protein was measured in terms of the absorbancy at 278 mμ. The absorbancies of the initial solutions were 5.50, 6.00 and 8.95 for BPA, edestin and serum respectively. The results are presented in Fig. 2.

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* The valuable help given by Mr R. S. RUFFIN in the construction of the apparatus is gratefully acknowledged.

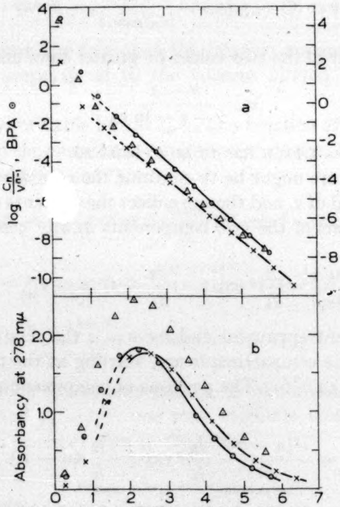

Fig. 2. Outflow concentration curves obtained with bovine plasma albumin (BPA), edestin and human serum.

We assume that the data can be represented by the model

$$c_n = \frac{m_A}{(n-1)!}\left(\frac{f}{v_0}\right)^n v^{n-1} \exp\left(-fv/v_0\right) \exp\left(\varepsilon\right) \tag{12}$$

where n is a known constant, m_A and f/v_0 are unknown parameters requiring estimation, and ε is a random error supposed to be normally distributed with expectation zero and constant variance σ^2.

Let

$$x = v$$

$$y = \ln \frac{c_n}{v^{n-1}}$$

$$\alpha = \ln \frac{m_A(-\beta)^n}{(n-1)!}$$

$$\beta = -f/v_0$$

Then (12) becomes

$$y = \alpha + \beta x + \varepsilon,$$

a simple linear model. Maximum likelihood estimates are found by the method of least squares to be

$$\hat{\beta} = \frac{N\Sigma xy - (\Sigma x)(\Sigma y)}{N\Sigma x^2 - (\Sigma x)^2}$$

$$\hat{\alpha} = \frac{1}{N}(\Sigma y - \hat{\beta}\Sigma x)$$

where N is the number of experimental observations. Hence maximum likelihood estimates of m_A and f/v_0 are given by

$$\widehat{f/v_0} = -\hat{\beta}$$

$$\hat{m}_A = \exp\,[\hat{\alpha} + \ln\,(n-1)! - n \ln\,(-\hat{\beta})]$$

Fig. 2a shows the plot of $\log(c_n/v^{n-1})$ against v for three experiments along with the lines of best fit. The early points in the curves are not expected to follow the theory because of the time lag for establishment of the steady state of liquid flow at all membranes. It can be seen that both BPA and edestin behave like single species, that is, there is no curvature, although ultracentrifugation* indicated 13 % and 2 % of faster sedimenting components in edestin. On the other hand, curvature was quite apparent in the case of serum, thus clearly reflecting its heterogeneous character. The estimates of f/v_0 were 1.78 and 1.63 for BPA and edestin respectively.

Fig. 2b shows c_n itself plotted against v. Again the fitted curves are shown. The description of the experimental data by equation (3) is seen to be adequate.

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A SIMPLE MEMBRANE FRACTIONATION METHOD FOR DETERMINING THE DISTRIBUTION OF RADIOACTIVITY IN CHEMICAL FRACTIONS OF *BACILLUS CEREUS*

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SUMMARY

A method has been devised for the rapid determination of the distribution of a labeled precursor in the chemical fractions of *Bacillus cereus*. The procedure involves the extraction of the cell by selective reagents, followed by filtration through collodion membranes. The distribution was then calculated by difference. By the use of suitable radioactive precursors for protein, DNA, and RNA, it was possible to examine and adapt the classical fractionation methods of SCHMIDT AND THANNHAUSER and of SCHNEIDER to the membrane filter method. Complete fractionations were carried out simply and reproducibly on a few milliliters of bacterial suspension, containing less than 0.5 mg dry weight of material.

INTRODUCTION

Collodion membrane filters have been used for several years for the filtration of dilute suspensions of bacteria, particularly in Public Health Analysis[1] and more recently as a rapid and convenient method of plating labeled organisms for radioassay[2–5]. BRITTEN, ROBERTS AND FRENCH[3] measured the incorporation of radioactive amino acids into the acid-soluble fraction of *Escherichia coli* by comparing the radioactivity on the membrane filter after filtration of the intact bacteria with that after filtration of bacteria washed with cold TCA. By this means incorporation of radioactivity into a chemical fraction could be measured without the actual isolation and plating of that fraction.

This principle of measuring radioactive incorporation indirectly, by difference, is exploited in this paper to measure the radioactivity extracted from *Bacillus cereus* by ethanol, by hot TCA and by NaOH. By using characteristic radioactive labels for RNA, DNA and protein, it has been possible to study the differential extraction of these materials with great accuracy and simplicity, and hence to find suitable conditions for their separation. The chemical fractionation was based on the methods

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; TCA, trichloroacetic acid.

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of SCHMIDT AND THANNHAUSER[6], SCHNEIDER[7], and ROBERTS, COWIE, ABELSON, BOLTON AND BRITTEN[8]. Characteristic labels included [8-^{14}C]guanine (for the purine bases of RNA and DNA), [2-^{14}C]uracil (for the pyrimidine bases of RNA and DNA), [Me-^{14}C]thymidine (for the thymine of DNA), and [1-^{14}C]leucine and [1-^{14}C]valine (for the respective amino acids of protein[9]).

MATERIALS AND METHODS

Bacterial growth

Radioactive bacterial suspensions were prepared by adding tracer to exponentially growing cultures of *Bacillus cereus*, strain 569H, grown in a salts-casein hydrolysate medium, as used in this laboratory[10]. The tracer was added to suspensions containing approximately 10^8 cells per ml and the cultures harvested when the cell number had doubled. More concentrated bacterial suspensions were difficult to filter through the membranes. Sufficient tracer was added to the suspension to obtain a final radioactivity of 500–2000 counts/min in the cells in 1 ml of medium.

Radioactive materials

Uniformly labeled D-glucose, [2-^{14}C]glycine, [8-^{14}C]guanine, DL-[1-^{14}C]leucine, DL-[3-^{14}C]phenylalanine and DL-[1-^{14}C]valine were purchased from the Isotopes Specialties Company, Burbank, California. [Me-^{14}C]thymidine was kindly furnished by the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Maryland. Uniformly labeled L-arginine was ordered from Nuclear Chicago Corp., Chicago, Ill.; DL-[2-^{14}C]lysine came from Tracerlab, Waltham, Mass., and [2-^{14}C]uracil was prepared in these laboratories[11]. All of the labeled compounds were tested for radiopurity prior to use.

Methods of fractionation

Centrifugation: 30 ml of bacterial suspension were mixed with 4.3 ml of 40% (w/v) TCA, kept in ice for 15 min and sedimented in a refrigerated centrifuge for 20 min at 10,000 × *g*. The supernatant solution, containing a mixture of the acid-soluble fraction and growth medium, was discarded. The sediment, after one washing with cold 5% TCA (w/v) was taken as the "cold TCA residue". This residue was heated at 70° for 30 min in 70% (v/v) ethanol, then in ethanol–ether mixture (1:1, v/v) at 40° for 10 min, and finally washed with ether at room temperature. The combined ether and ethanol extracts were concentrated and plated as the "ethanol extract". The dry residue from this extraction was heated in 3.0 ml of 5% (w/v) TCA at 100° for 30 min. The sediment, after cooling in ice for 15 min, and centrifuging, was washed with 5% (w/v) TCA at 0°, and the combined hot TCA extract and washings were shaken with ether 6 times in order to remove TCA. The aqueous phase, containing the decomposed nucleic acids, was then plated as the "hot TCA extract". The residue from this hot TCA extraction, containing mainly proteins ("hot TCA residue" or "total protein fraction") was plated after hydrolysis in 3.0 ml of 6.0 *N* HCl in a pressure cooker at 8 lb./in^2 for 2.5 h, followed by removal of the HCl by evaporation *in vacuo* over NaOH and P_2O_5.

Membrane filtration: Membrane filters, 27 mm in diameter, were obtained from Schleicher and Schüll Co., Keene, New Hampshire. According to the manufacturers, the "coarse" and "medium" porosity membranes had an average pore size of 0.4 μ and 0.25 μ, respectively. Medium membranes were used in this study except where indicated. The membranes were dipped in water and placed on a stainless steel grid, (designed and kindly provided by the Carnegie Institution of Washington, Washington, D.C.) attached to an aspirator. 2.0 ml of the bacterial suspension was added to a series of test tubes containing either ice-cold saline, 10 % TCA, ethanol or NaOH, as described below. The resulting suspension then was poured onto the membrane and washed with three 5-ml samples of appropriate washing fluid. No further radioactivity was removed by extra washing. The membranes were then fastened onto 1.25 inch-diameter aluminum planchets with a thin layer of rubber cement, allowed to dry at room temperature, and counted in a Nuclear Chicago Automatic proportional gas-flow counter, using natural gas. Counts were continued until a standard error of less than 1 % was obtained. No correction was necessary for self-absorption.

RESULTS

Treatment with cold saline

To determine the radioactivity present in intact bacterial cells, an aliquot of a suspension of *Bacillus cereus* growing in the presence of a labeled precursor was added to an equal volume of ice-cold 0.9 % saline solution. The mixture was filtered immediately through membrane filters and was washed with additional saline. Growth medium could be used to substitute for the saline with identical results.

Measurements with intact bacteria occasionally showed slight variability because of some continued uptake of radioactivity until the filtration was finished. As a standard for comparison, therefore, the residual radioactivity after treatment with cold TCA was preferred.

Treatment with cold TCA

To remove the acid-soluble fraction, suspensions of *B. cereus* were mixed with an equal volume of 10 % TCA, and the resulting mixture was filtered after 15 min and washed with 1 % TCA. No marked difference was found between suspensions kept at 0° or at room temperature. The results of the other fractionation treatments were expressed as per cent of the radioactivity in the cold TCA residue.

The difference between the radioactivity of the bacterial samples filtered after addition to saline and TCA represented the acid-soluble fraction. Usually only 5 to 10 % of the total radioactivity of the cells grown in the presence of the labeled compounds was associated with this fraction.

Treatment with hot TCA

Bacteria grown in the presence of radioactive valine, leucine, thymidine and guanine were mixed with an equal volume of 10 % TCA, and heated at 100° for various periods. After cooling, the suspensions were filtered and washed with 1 % TCA. This extraction, carried out under conditions similar to those described by SCHNEIDER[7], produced results shown in Fig. 1. There was virtually no extraction of radioactivity from bacteria labeled with valine, whereas there was complete removal of activity from cells grown with [^{14}C]guanine. A small amount of radioactivity was released slowly from [1-^{14}C]leucine labeled bacteria. The extraction of thymidine was incomplete after 30 min, but further extraction removed no more radioactivity. 30 min at 100° was therefore used as the optimal period for extraction.

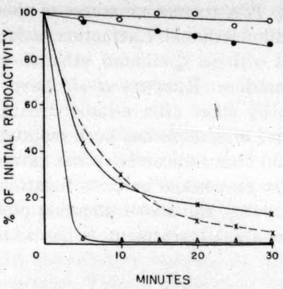

Fig. 1. Extraction of radioactive bacteria with hot TCA. ○—○, [1-^{14}C]valine; ●—●, [1-^{14}C]leucine; △—△, [8-^{14}C]guanine; ×—×, [Me-^{14}C]thymidine (medium porosity membrane); ×---×, [Me-^{14}C]thymidine (coarse porosity membrane). Bacteria grown with various isotopic precursors were mixed with equal volumes of cold 10% TCA. Suspensions were then placed in a water bath at 100° for various periods, filtered and counted for radioactivity. Medium porosity filters used except as indicated.

Treatment with hot TCA clearly differentiated between incorporation into nucleic acids and total proteins (Table I). Thus radioactivity after labeling with various amino acids was found predominantly in the residue after extraction with hot TCA, whereas radioactivity from RNA precursors was recovered quantitatively in the hot TCA extract. [2-^{14}C]glycine, a precursor of both nucleic acids and proteins, was distributed more or less evenly between the extract and residue.

Treatment with ethanol

Experiments on the "lipid" fraction were limited by the fact that the collodion membrane filters dissolved in some organic solvents (*e.g.*, chloroform, acetone, and ethanol–ether (1:1)). They did not dissolve in 65% (v/v) ethanol, however.

TABLE I

EXTRACTION OF RADIOACTIVITY WITH HOT TCA OR NaOH FROM *Bacillus cereus* LABELED WITH VARIOUS TRACERS

Results expressed as percent of radioactivity in the cold TCA extracted residue. After NaOH treatment the suspensions were acidified with HCl and TCA before filtering.

Radioactive precursor	*Percent extracted by 5% TCA at 100° for 30 min*	*Percent extracted by 0.5 N NaOH at 37° for 2 h*
[8-^{14}C]guanine	99.9	94.1
[2-^{14}C]uracil	99.9	93.0
[Me-^{14}C]thymidine	95.9*	2.5
[2-^{14}C]glycine	61.3	53.6
[^{14}C]arginine (uniformly labeled)	9.8	0.0
[1-^{14}C]leucine	6.0	1.0
[2-^{14}C]lysine	6.1	0.0
[1-^{14}C]valine	5.0	0.0
[3-^{14}C]phenylalanine	3.0	—

* Results with coarse membrane filters. Medium filters showed 85–90% extraction.

Bacterial suspensions were therefore added to 2 volumes of ethanol, and the mixture heated at 70° for 30 min. After cooling the suspension and filtration, the filter was washed with 5 % TCA to remove traces of the acid-soluble fraction that were not extracted by the dilute ethanol. Extraction under these conditions produced similar results to treatment with 70 % ethanol, ethanol–ether and ether in the conventional fractionation procedure. ROBERTS *et al.*[8] have reported little further extraction of bacterial lipids by ether after ethanol treatment. The lipid content of cell walls from Gram-positive organisms has been reported to be lower than that of Gram-negative bacteria[12] and little radioactivity was extracted from *B. cereus* (Gram-positive) by either the filter membrane or centrifugation method. In studies with *E. coli* (Gram-negative), however, the above filtration procedure indicated considerable extraction of lipids after alcohol treatment, as has been found after centrifugation techniques[8].

Treatment with NaOH

An aliquot of the bacterial suspension grown with [Me-^{14}C]thymidine was incubated at 37° for 15 h in *N* NaOH (according to SCHMIDT AND THANNHAUSER[6]) and the alkaline suspension filtered onto the membrane and washed with water. Radioactivity results were identical to those after cold TCA treatment, indicating that the alkali had not extracted any radioactivity from the cold TCA residue. Treatment with 5 % TCA at 100° for 30 min, after the alkali digestion, however, removed the bulk of the activity. Thus DNA apparently was not extracted by the alkali treatment.

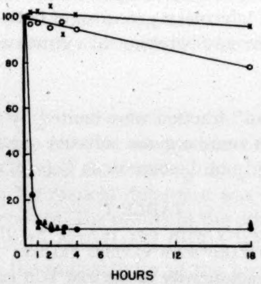

Fig. 2. Extraction of radioactive bacteria with NaOH at 37°. ×—×, [Me-^{14}C]thymidine; ○—○, [1-^{14}C]valine; △—△, [8-^{14}C]guanine; ●—●, [2-^{14}C]uracil. Bacteria grown with various isotopic precursors were incubated in 0.5 *N* NaOH at 37° for various periods and the suspensions were acidified and assayed for radioactivity.

In contrast to the results with [Me-^{14}C]thymidine, the alkali treatment, under similar conditions, of bacteria labeled with [8-^{14}C]guanine or [2-^{14}C]uracil removed about 95 % of the radioactivity. Such a result would be anticipated, for alkali treatment is known to hydrolyze RNA to mononucleotides[6] which would be readily extracted from the cells. Since the RNA/DNA ratio in exponentially growing *Bacillus cereus* is 10 or 15:1[13,14], at least 90 % of the radioactivity with [8-^{14}C]guanine and [2-^{14}C]uracil would be expected to be in the RNA. The residual activity would be in DNA which, as in the thymidine experiment, would be unextracted by NaOH.

The residue from NaOH-treated bacteria was treated with *N* HCl at 100° for 1 h, followed by chromatography in isopropanol–conc. HCl (680 ml/176 ml made up to 1 l, adapted from ref. [15]). Adenine and guanine were readily detected but no pyrimidine nucleotides were observed, indicating that RNA had been separated from DNA.

When this alkali treatment was used with bacteria labeled with [1-^{14}C]leucine, considerable breakdown of protein was noted. In a search for milder conditions of alkaline hydrolysis, 1.5 to 2 h incubation with 0.5 *N* NaOH at 37° was found to be sufficient for maximal extraction of radiocarbon from bacteria labeled with [8-^{14}C]-guanine or [2-^{14}C]uracil, during which time there was little release of activity from bacteria labeled with [1-^{14}C]valine or [Me-^{14}C]thymidine (Fig. 2). It was the general procedure to acidify the alkaline suspension with HCl and TCA at 0° prior to filtration to reprecipitate any protein dissolved by the NaOH. These conditions, therefore, were adopted for the specific and quantitative removal of RNA with the minimum loss of protein into the alkaline extract. Table I (Column 2) shows the results under these conditions with several radioactive materials, and it can be seen that the extraction of the radioactive amino acids was negligible.

Limitation of the technique

It was observed in experiments with certain tracers that far more radioactivity was extracted by hot TCA treatment than could be accounted for by the extraction of nucleic acid alone. Chromatography of hot TCA extracts of bacteria labeled with [1-^{14}C]alanine followed by radioautography revealed that in addition to the nucleic acid products, much radioactivity was associated with unknown compounds. Alkali treatment of cells released small amounts of a product different from nucleic acids. In view of the general stability of protein during hot TCA or alkali treatments, it was concluded that in all likelihood, the product arose from decomposition of cell walls, and that cell wall precursors could interfere with the present procedure. The differential effect of hot TCA and alkali treatments were most pronounced when cells were grown with [4-^{14}C]aspartic acid, [2-^{14}C]glutamic acid, [3-^{14}C]serine and [1-^{14}C]-alanine[9]. These amino acids have been shown recently to contribute radiocarbon to bacterial cell walls. It is important to realize, however, that any conventional fractionation method suffers from the same difficulties as the membrane filtration technique described here.

Another limitation of the technique was observed while using [^{35}S] inorganic sulfate, which contained a trace of impurity that adsorbed onto the membrane filters and produced erratic counts.

Porosity of membranes

The same distribution patterns were obtained with coarse or medium membranes for most of the isotopic precursors examined, although absolute counts usually were 5 % higher with medium membranes. With labeled thymidine, however, it was found that higher activities were obtained when the hot TCA residue was filtered on medium filters than on coarse (Fig. 1). This difference probably was related to the heterogeneity in particle size produced by acid hydrolysis of DNA. Variation was also observed when cells labeled with [1-^{14}C]leucine were subjected to NaOH treatment and filtered on coarse membranes. Only the use of medium membranes permitted reproducible recoveries with this particular radioisotope.

Final fractionation scheme

On the basis of the above experiments, the following scheme has been employed to demonstrate the distribution of radioisotopes in the chemical fraction of the microorganisms (Table II).

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN CHEMICAL FRACTIONS AS DETERMINED BY THE MEMBRANE FILTER METHOD

Results expressed as a percentage of the radioactivity in the cold TCA residue (filter No. 2). See text for details of membrane filter fractionation.

Filter No.	*Radioactive tracer* *[1-^{14}C]leucine*	 *[8-^{14}C]guanine*	 *[Me-^{14}C]thymidine*	
1	105.9	106.3	108.3	
2	(100.0)	(100.0)	(100.0)	
3	96.0	0.1	4.5*	
4	95.8	97.5	96.2	
5	99.0	5.9	96.2	
Fraction				*Content*
Cold TCA extract	5.9	6.3	8.3	Acid-soluble
Ethanol extract	4.2	2.5	3.8	Lipid
Hot TCA extract	4.0	99.9	95.5	RNA, almost all DNA, +?
Hot TCA residue	96.0	0.1	4.5	"Total protein" and trace of DNA
NaOH extract	1.0	94.1	3.8	RNA + ?
NaOH residue	99.0	5.9	96.2	DNA and "total protein"

* Result with coarse membranes. Medium filters gave value of 10–15%.

Add 2.0-ml aliquots of the bacterial suspensions of labeled cells of *B. cereus* to test tubes containing the following reagents: (a) 2.0 ml 0.9% NaCl at 0°. Filter immediately and wash with 0.9% NaCl (filter No. 1, intact cells). (b) 2.0 ml 10% (w/v) TCA. Allow to stand in ice for 15 min, filter and wash with 1% TCA (filter No. 2, cold TCA residue). (c) 2.0 ml 10% TCA. Heat at 100° for 30 min, cool in ice for 15 min, filter and wash with 1% TCA (filter No. 3, hot TCA residue). (d) 4.0 ml 95% ethanol. Heat at 70° for 30 min, cool in ice for 15 min, filter, wash with 5% TCA and then 1% TCA (filter No. 4, alcohol insoluble residue). (e) 0.2 ml 5.5 *N* NaOH.

TABLE III

COMPARISON BETWEEN FRACTIONATION OF LABELED BACTERIAL CELLS BY CENTRIFUGATION AND MEMBRANE FILTER TECHNIQUES

Results expressed as percent of the radioactivity in the cold TCA extracted residue. See text for details of the two fractionation schemes.

Tracer	*Method*	*Ethanol extract*	*Hot TCA extract*	*Hot TCA residue*
[1-^{14}C]leucine	Membrane filtration	4.2	4.0	96.0
	Centrifugation	2.5	4.4	93.0
[^{14}C]glucose (uniformly labeled)	Membrane filtration	—	57.8	42.2
	Centrifugation	—	56.5	42.9
[2-^{14}C]glycine	Membrane filtration	5.6	61.3	38.7
	Centrifugation	5.9	56.8	37.1

Incubate at 37° for 2 h with occasional shaking, cool in ice while adding 0.2 ml 6 *N* HCl and 2.5 ml 10 % TCA, keep at 0° for another 15 min, filter and wash with 1 % TCA (filter No. 5, NaOH insoluble residue).

The radioactivity in the various extracts can then be calculated by difference: cold TCA extract, 1–2; ethanol extract, 2–4; hot TCA extract, 2–3; NaOH extract, 2–5.

A comparison of the membrane filter method and the conventional method involving centrifuging and the direct plating of extracts is shown in Table III. It is apparent that essentially identical results are obtained with the two methods.

DISCUSSION

The results reported above have some bearing on the effectiveness of separation of fractions by the techniques of SCHNEIDER[7] and of SCHMIDT AND THANNHAUSER[6]. Treatment of *Bacillus cereus* with TCA at 95° for 15 min, as recommended by SCHNEIDER, was insufficient to remove all the DNA as measured by [Me-^{14}C]thymidine (Fig. 1). It can also be seen in Fig. 1 that there was a small but steady release of radioactivity from organisms labeled with [1-^{14}C]leucine (possibly because of the release of small peptides containing leucine) that continued well after the extraction of radioactivity from a culture labeled with [8-^{14}C]guanine had ceased. In bacteria, in particular, it is erroneous to assume that only nucleic acid is extracted by hot TCA.

The classical SCHMIDT AND THANNHAUSER[6] extraction with *N* KOH or NaOH at 37° for 15 to 18 h effectively removed RNA in the present studies. It is interesting, however, that DNA was not dissolved out of the cells by these conditions, since direct filtration of alkaline suspensions of the bacteria (without acidification) led to the complete retention of [Me-^{14}C]thymidine activity on the filter. DUNN AND SMITH[16] have also reported difficulty in extracting DNA from *Bacillus cereus* with alkali, although this procedure did extract DNA from other cells. In a detailed chemical fractionation of the same organism, FITZ-JAMES[17] also reported that the bulk of the DNA remains within the cell after treatment with *N* KOH at 37° for 18 h.

MAURITZEN, ROY AND STEDMAN[18] and MOULÉ[19] have shown that the "RNA" fraction in the SCHMIDT AND THANNHAUSER method is contaminated with protein breakdown products. The steady release of activity from bacteria labeled with [1-^{14}C]-valine in 0.5 *N* NaOH, as shown in Fig. 2, would confirm this observation. Use of short periods of alkali treatment (2–3 h) greatly reduced this effect, without preventing the quantitative removal of RNA.

The disadvantages and advantages of the membrane filter method may be briefly indicated. As the procedure is based on the measurement of radioactivity by difference, errors in one membrane filter will reflect themselves in more than one fraction. Also, it is not possible to determine specific activities since the fraction is not directly isolated. It is advisable to carry out the determinations in duplicate, to eliminate possible small errors due to excessive pore size and to ascertain that the radioactive material is not adsorbed on the membrane (a rare occurrence). Otherwise, the method is rapid, simple, most reproducible, and can be carried out on small quantities of material. About 0.3 mg dry weight of cells was usually used in this study. With ^{14}C, no self-absorption correction was required to 3 times that amount, and all the sediment is located on top of the membranes. With tritium compounds, on the other hand, self-absorption plays such a large role that the fractionation procedure would have to be carried out on minute quantities. Once infinite thickness values are reached, total counts on a filter are related to the product of the specific activity values and the weight of the sample. Such a procedure has been described[20].

The only manipulations involved are the pipetting of 1 volume into a test tube, heating the test tube and filtering the contents. Because of the simplicity of the manipulation, the errors are far less than in methods that involve sedimentation, resuspension and transfer of precipitates, leading to cross-contamination and loss of material. The method can also be used for ultramicro quantities. All the samples are plated with identical geometry which could not be readily duplicated by plating in the customary manner. Practically all the reagents have been removed by the washing with 1 % TCA and do not interfere with the counting. The principle of determining incorporation by measuring the loss of radioactivity after specific extraction procedures is of general application and the authors feel that the filtration method could be readily used with biological materials other than *Bacillus cereus*, if properly adapted.

After the completion of the present work, a microassay method for nucleic acid synthesis was described by HANAWALT[21]. This procedure involved filtration of labeled *E. coli* on membrane filters and treatment of the membranes with various reagents. The method was designed for ^{32}P, an isotope where there is little self absorption. Because the method required solution and reprecipitation of the membranes themselves it may not be as versatile as the present procedure, especially for isotopes emitting softer radiation.

ACKNOWLEDGEMENT

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THE EFFECTS OF CARBON DIOXIDE AND HYDROGEN ION ON SODIUM TRANSPORT IN ISOLATED FROG SKIN*

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SUMMARY

A detailed study has been made of the effect of CO_2 and hydrogen ion on the sodium ion transport system of isolated frog skin. The short-circuit technique has been used as a measure of the net sodium flux, and open circuit potential measurements were made as well.

The sodium ion flux was investigated as a function of sodium ion concentration in the solution bathing the external surface at a variety of pH's in the range 6–8.4. These data were subject to analysis in terms of a model previously proposed and the inhibition of the sodium transport system by H^+ was found to be interpretable as non-competitive.

The sodium ion flux was also investigated following step changes in pH of the bathing solutions as well as changes in buffer system at the same pH. The resulting transient changes in sodium ion flux are interpreted with the aid of a simple model mechanism which accounts for the observed differences in effect between a $CO_2-HCO_3^-$ buffer and phosphate buffer in terms of the relative rapid diffusion of CO_2 leading to local changes in H^+ concentration at the site of the transport system.

INTRODUCTION

The influence of hydrogen ion on the active sodium transport system of the isolated frog skin has been studied by a number of investigators[1–4]. All of these studies have shown that hydrogen ion inhibits the active sodium transport at concentrations greater than 10^{-8} *M*. This inhibition is most pronounced when the pH of the solution bathing the inner surface of the skin is lowered. If the pH variation is brought about only in the solution bathing the external surface, a lower value of pH is necessary to produce comparable inhibition. Another fact of interest has been the observation that if pH variations are brought about by alteration of the carbon dioxide in a CO_2-HCO_3-buffered Ringer's solution, then the effect on the sodium transport

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system is produced more rapidly than when comparable pH changes are made with a phosphate-buffered Ringer's solution.

In this paper are reported some further observations on the influence of hydrogen ion on the sodium transport system of the isolated skin of *Rana pipiens*. The results of the rapid responses of the sodium transport system to variations in CO_2 are interpreted in quantitative terms based on a local change in pH within the skin due to the relative rapid diffusion of CO_2. The role of hydrogen ion itself on the sodium transport system is given quantitative interpretation in terms of a simplified model.

METHODS

The net active sodium flux in the isolated skin has been measured as the short-circuit current according to the method of USSING AND ZERAHN[5]. Lucite chambers giving an effective skin area of 2.9 cm^2 and a volume of 6 ml per side were employed. The electrodes in each chamber were as has been previously described[6]. In general duplicate chambers were used and the abdominal skin of a single frog was divided into approximately symmetrical halves. The composition and pH of the solutions with which the skin surfaces were bathed are given in Table I. All chemicals were reagent grade. The choline chloride was obtained from Merck. The solutions bathing the inside skin surface were invariably the sodium-containing solutions. To obtain varying sodium ion concentrations in the solutions bathing the external surface, varying quantities of the sodium containing solution and the corresponding choline containing solution were mixed. The solutions bathing both surfaces of the skin were identical except for the reciprocal variations of the sodium and choline ion concentrations in the solutions bathing the external surface. Those solutions equilibrated with air were not further aerated while in the chambers, but instead were changed

TABLE I

COMPOSITION OF RINGER'S SOLUTIONS. CONCENTRATIONS IN mmoles/l

	NaCl	*Choline chloride*	*$KHCO_3$*	*K_2HPO_4* *KH_2PO_4*	*$CaCl_2$*	*Equilibrated with*	*pH*
Sodium Ringer's solution	115		2.5		1	air	8.4
Choline Ringer's solution		115	2.5		1	air	8.4
Phosphate buffer (sodium)	110–113*			6		air	6.5–7.1
Phosphate buffer (choline)		110–113*		6		air	6.5–7.1
Bicarbonate buffer (sodium)	116–99*		2.5–20			95% O_2 5% CO_2	6.2–7.1
Bicarbonate buffer (choline)		116–99*	2.5–20			95% O_2 5% CO_2	6.2–7.1

* Concentration to give total of 0.238 *M*.

at intervals not greater than 10 min. Those solutions equilibrated with 5 % CO_2–95 % O_2 were gassed while in the chambers by means of a fine capillary below the solution surface. In changing from one type of solution bathing the skin to another, the chambers were first rinsed twice with the new solution. All experiments were made at the ambient temperature of 20–24°.

RESULTS

In the first group of experiments the net sodium ion flux, measured as the short-circuit current, I_0, was determined as a function of the sodium concentration in the solution bathing the external skin surface. The measurements were made at several different pH values. Data from a typical experiment utilizing a bicarbonate buffer system are given in Table II together with some derived results. In Fig. 1-A is seen a plot of C'_{Na}/I_0 *vs.* C'_{Na}, where C'_{Na} represents the concentration of Na^+ in the solution bathing the external surface. According to the model mechanism of transport previously developed[6] the data plotted in this manner should lead to a straight line whose slope is the reciprocal of the theoretical maximum flux, $\Omega_{NaX}X_t$, where Ω_{NaX} is related to the mobility of NaX, the hypothetical carrier complex,

TABLE II

EXPERIMENTAL DATA AND DERIVED RESULTS

5% CO_2–HCO_3^- buffer at pH 7.1, 6.8, and 6.5

pH	C'_{Na} %	$\Delta\phi_m$ *mV*	I_0 *μA cm⁻²*	C'_{Na}/I_0	λ_m *mho·10⁻⁴ cm⁻²*	*K* %	$\Omega_{NaX}X_t$ *μA cm⁻²*	$\lambda_m(K+C'_{Na})$ % *mho 10⁻² cm⁻²*	λ_p *mho 10⁻⁴ cm⁻²*	λ_t *mho 10⁻⁴ cm⁻²*	ϕ_t *mV*
8.4	100	33	28.2	3.54	8.6	14	32.3	9.7	6.0	2.6	111
	50	30	26.9	1.86	9.0			5.7			
	10	19	13.8	0.72	7.3			1.7			
	5	12	8.3	0.60	6.9			1.3			
	2	8	4.8	0.41	6.0			1.0			
7.1	100	28	15.5	6.45	5.5	12	17.7	6.2	4.7	0.9	178
	50	26.5	14.6	3.43	5.5			3.4			
	10	15	8.3	1.20	5.5			1.2			
	5	11	5.2	0.96	4.7			0.8			
	2	6.5	2.9	0.69	4.5			0.6			
6.8	100	18	9.6	10.4	5.3	15	11.1	6.1	3.3	2.0	48
	50	17	8.8	5.68	5.2			3.4			
	10	8.5	3.6	2.78	4.2			1.1			
	5	4	1.7	2.94	4.2			0.8			
	2	1	0.4	5.71	3.5			0.6			
6.5	100	8	4.5	22.2	5.6	16	4.7	6.5	3.1	2.5	18
	50	6.5	3.4	14.5	5.3			3.5			
	10	2	0.7	14.3	3.5			0.9			
8.4	100	30	22.4	4.46	7.5	16	26.2	8.7	5.8	1.7	130
	50	27	20.4	2.45	7.6			5.0			
	10	15	9.8	1.02	6.5			1.7			
	5	10	6.2	0.81	6.2			1.3			
	2	5	2.9	0.69	5.8			1.0			

and X_t represents the total concentration of this carrier, both unassociated and associated with Na+. The intercept at $C'_{\mathrm{Na}} = 0$ is $K/\Omega_{\mathrm{NaX}}X_t$ and the intercept at $C'_{\mathrm{Na}}/I_0 = 0$ is $-K$, where K is the stoichiometric dissociation constant of the carrier complex. Analysis of the data as plotted in Fig. 1-A indicates that on altering the pH of the bathing solutions only $\Omega_{\mathrm{NaX}}\, X_t$ is altered, becoming smaller with decreasing pH, while the K remains nearly constant. This may be interpreted in terms of this model mechanism as a noncompetitive type of inhibition of the sodium ion transport system due to hydrogen ion, at least in the pH range encompassed by these experiments; and this would indicate that there is no significant association of the free carrier substance, X, with hydrogen ion at a site in competition with Na ion.

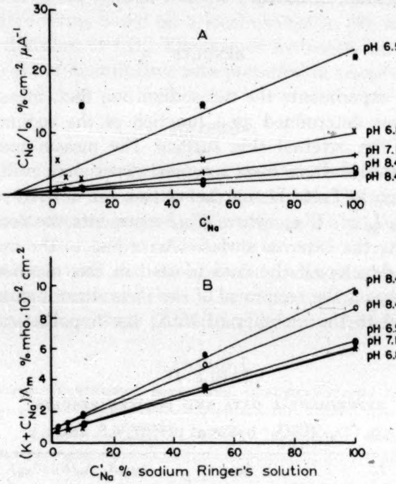

Fig. 1. The data of Table II plotted for analysis by the model system of SNELL AND LEEMAN[6].

In Fig. 1-B are seen plots of the function, $\lambda_m(C'_{\mathrm{Na}} + K)$, *vs.* C'_{Na}, where λ_m represents the measured conductance of the skin. According to the model mechanism previously developed the data plotted in this manner should lead to a straight line whose intercept at $C'_{\mathrm{Na}} = 0$ is the product $\lambda_p K$, where λ_p is the passive conductance component of total conductance. With the aid of the equivalent electrical circuit of the skin, the active transport conductance, λ_t, and the active transport potential, ϕ_t, are obtained. The values of the various parameters at the different experimental pH's are listed in Table II.

In the second group of experiments to be reported, difference in the effects of a phosphate-buffered solution and a bicarbonate–carbon dioxide-buffered solution on the sodium ion transport system are investigated. Results typical of these experiments are seen in Fig. 2 in which the short-circuit current is plotted *vs.* time and changes in experimental conditions are indicated. The broken line represents the data of the control half-skin maintained throughout with sodium Ringer's solution at pH 8.4 bathing each surface. The solid line represents the data of the experimental half-skin. After an initial period of stabilization with sodium Ringer's solution, shifts are made from a bicarbonate–carbon dioxide-buffered solution to a phosphate-buffered solution at the same pH. It may be seen that immediately following this change there is observed a large transient increase in the short-circuit current. It may also be seen that these transients are produced at three different pH values of the bathing solutions, 7.1, 6.8, and 6.5. The transient curves all appear to have essentially the same form characterized by a rapid rise in the short-circuit current followed by a slower decline. This fact suggests that if the primary inhibitor of the sodium transport system is the hydrogen ion itself, it is possible that due to a differential rate of CO_2 diffusion and HCO_3^- diffusion, a transient change in H^+ concentration could occur at the site of the transport mechanism[7]. In attempting to obtain a quantitative explanation of these transients based on this line of reasoning, several points of possible significance emerged.

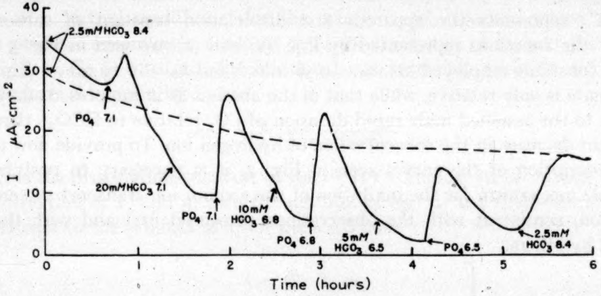

Fig. 2. The short-circuit current of an experimental (solid line) and control skin (broken line) plotted *vs.* time. The arrows indicate points where changes were made in the composition of the bathing solutions.

Consider the frog skin to be represented by a thin plane phase of unit area and with thickness, Δx, bounded on each side by large well-stirred phases representing the bathing solutions. An approximate solution to the flux equation would indicate that the concentration of a constituent in the plane phase will vary exponentially with time following a step change in the concentration of a component in the adjacent phases. With the boundary conditions of C° and C^∞ representing the initial and final concentrations of the constituent respectively, the concentration within the plane phase, C, at the time, t, is given as

$$C = C^\infty + (C^\circ - C^\infty)e^{-kt} \tag{1}$$

where k represents the rate constant. The rate constant is related to the diffusion coefficient, D, by the relation $k = D/(\Delta x)^2$. Eq. (1) is assumed as an approximate description valid for either CO_2 or HCO_3^-, expressing the concentration of these constituents within the skin as a function of time, following a step change of their concentrations in the bathing solutions. The subscripts, c and b, are used to denote these two substances. Since the initial and final pH in any one step change in the bathing solutions are the same, it follows from equilibrium considerations that $C^\circ_c/C^\circ_b = C^\infty_c/C^\infty_b$. Thus the hydrogen ion concentration within the skin, C_h, assumed to be in equilibrium with HCO_3^- and CO_2 during the transient period is approximated by the equation

$$C_h = K'_1 \frac{C_c^\infty + (C_c^\circ - C_c^\infty)\, e^{-k_c t}}{C_b^\infty + (C_b^\circ - C_b^\infty)\, e^{-k_b t}} \tag{2}$$

where K'_1 represents the apparent first dissociation constant of carbonic acid. Plots of the functions represented by Eqs. (1) and (2) are seen in Fig. 3 in which the rate constants employed are $k_c = 0.36 \text{ min}^{-1}$ and $k_b = 0.10 \text{ min}^{-1}$. The scale of the ordinate is only relative, while that of the abscissa is in minutes. It may be seen that due to the assumed more rapid diffusion of CO_2 relative to HCO_3^- there occurs a transient decrease in the concentration of hydrogen ion. To provide now a quantitative description of the curves seen in Fig. 2, it is necessary to postulate some reasonable mechanism for the inhibition of the sodium ion transport system by hydrogen ion, consistent with the observations presented first and with the results obtained by others.

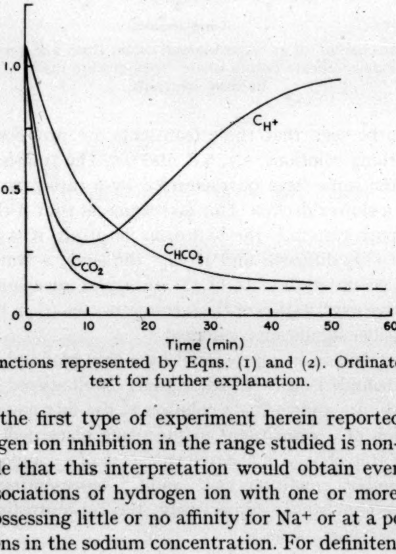

Fig. 3. Plots of the functions represented by Eqns. (1) and (2). Ordinate in relative units. See text for further explanation.

The results of the first type of experiment herein reported indicated that the nature of the hydrogen ion inhibition in the range studied is non-competitive. Nevertheless, it is possible that this interpretation would obtain even though there were actual reversible associations of hydrogen ion with one or more constituents of the transport system possessing little or no affinity for Na^+ or at a point remote from the influence of variations in the sodium concentration. For definiteness we may visualize a cyclic carrier transport system represented by the following scheme:

$$\begin{array}{rcl}
Na + X = NaX & \longrightarrow & NaX = X + Na \\
 & & + \\
 & & Y_1 + H = HY_1 \\
 & & + \\
 & & Y_2 + H = HY_2 \\
 & & + \\
 & & \cdots\cdots \\
 & & + \\
X = Z & \longleftarrow & Z = Y_n + H = HY_n \\
\text{external surface} & & \text{internal surface}
\end{array} \tag{3}$$

In this scheme X represents the carrier constituent formed from a constituent, Z, at the external physiologic surface of the membrane represented by the double vertical lines. The Y's represent those generalized substances which on reacting with X form Z. It is then postulated that any or all of the Y's may reversibly associate with hydrogen ion to form inactive complexes. The equal signs in (3) signify the existence of a virtual equilibrium for the reactions, whereas the arrows signify a diffusion process. Assuming a constant total concentration of each of the Y's, *i.e.* $C_{Y_i} + C_{HY_i} = C_i =$ constant, the fractional concentration available for reaction with X to form Z is given by

$$a_i = \frac{C_{Yi}}{C_i} = \frac{1}{1 + C_H/K_i} \tag{4}$$

where K_i is the dissociation constant of the i^{th} HY complex. In the steady state the rate of formation of Z, and in turn the net flux of Na^+ or the short-circuit current, is proportional to the product of all of the α's. Thus

$$I = I_0^m \prod_{i=1}^{n} a_i \tag{5}$$

where I_0^m is the proportionality constant and is the maximum short-circuit current. The combination of Eqs. (2), (4), and (5) gives

$$I_0 = I_0^m \prod_{i=1}^{n} \left[\frac{K_i}{K_i + K'_1 \dfrac{C_c^{\infty} + (\overset{\circ}{C}_c - C_c^{\infty})\, e^{-k_c t}}{C_b^{\infty} + (\overset{\circ}{C}_b - C_b^{\infty})\, e^{-k_b t}}} \right] \tag{6}$$

In applying Eqn. (6) to the experimental data shown in Fig. 2, it was found that the best overall fit could be obtained with $n = 2$. Furthermore, a fit, reasonably good, could be obtained employing a single value for the two K's. Only slight improvement in fit results with the use of two different values for the K's and was consequently avoided.

The experimental data shown in Fig. 2 are replotted on an expanded time scale in Fig. 4 at the three different pH values of the bathing solutions. The solid lines are the curves generated by Eqn. (6) with $n = 2$. The values for the other adjustable parameters are $K_1 = K_2 = 1.25 \cdot 10^{-7}$ moles l^{-1}, $k_c = 0.36$ min^{-1}, and $k_b = 0.10$ min^{-1}. It is evident from this figure that the assumed model system serves well to describe in quantitative terms the experimental findings.

DISCUSSION

It is apparent from the results of the first experiments reported above that the nature of the inhibition of the sodium transport system by H^+ is classically noncompetitive, at least with respect to Na^+ variations at the external surface, and within the range experimentally explored, pH 6.1–8.4. Thus there appears to be in this pH range no significant direct competition of Na^+ and H^+ for the transport mechanism. If competition occurs, our evidence suggests that it must occur at a point in the transport system where the short-circuit current cannot be significantly altered by variations in Na^+ concentration. When simultaneous variations of the Na^+ concentration are made in the solutions bathing both surfaces of the skin, as has been done in some of our experiments, the results obtained are almost identical to those obtained when the variation is made only in the solution bathing the external surface alone. The observed H^+ inhibition is completely reversible, a finding in agreement with those of USSING[1], and SCHOFFENIELS[3,4]. Both of these authors have interpreted their data, however, as indicative of a "competitive" inhibition, and have postulated a reversible association of H^+ with the sodium "carrier" component. Our observations, on the other hand, although consistent with a reversible association of H^+, indicate that this association must be either with some component of the transport system which is not to be identified as the sodium ion carrier, or if involving the carrier, at some site other than that for sodium, yet still effecting inhibition.

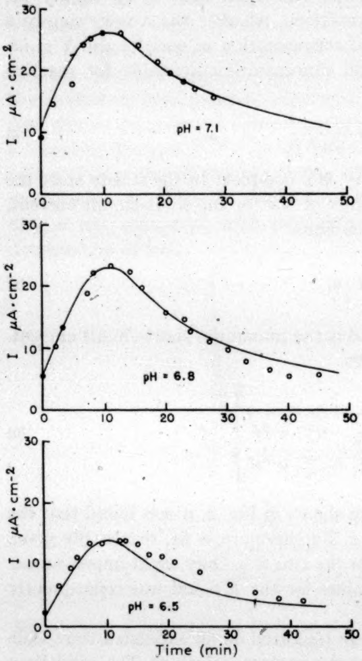

Fig. 4.

Fig. 4. Portions of the data (open circles) of Fig. 2 replotted on expanded time scale. The solid lines are the function represented by Eqn. (6) together with the appropriate boundary conditions.

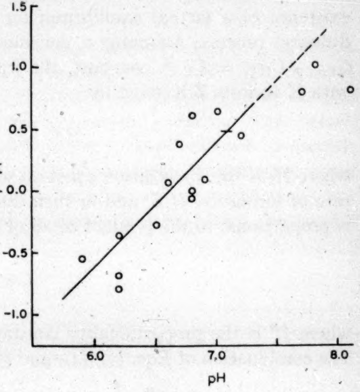

Fig. 5.

Fig. 5. Some of the data from SCHOFFENIELS[3] plotted to test the validity of $n = 2$. The variable, i, is the fractional short-circuit current.

The analysis of the results of the second group of experiments reported above are of interest in several respects. Although this analysis does not comprise in any way proof of the one possible mechanism assumed, the fact that there is a good degree of reasonableness in the values of the several parameters used to obtain fit to the experimental data does lend some support to the hypothesis. The fit of Eqn. (6) to the experimental data is highly sensitive to the value of n employed. Only integer values and rational fractions were tested, for only such values are interpretable in the model employed. A value of $n = 2$ as finally chosen, signifies that two constituents involved in the conversion of the carrier, X, to the postulated inactive form, Z, may be rendered inactive by their association with H^+. SCHOFFENIELS[3] has made some careful measurements of the effect of pH of the bathing solutions on the short-circuit current. He has plotted his data as the percentage of short-circuit current *vs.* the pH of the bathing solutions. Taking from his figure that data which refers to those experiments in which variations were made in the solution bathing only the inner surface or in the solutions bathing both the inner and outer surface, we have replotted it in a manner to test the value of $n = 2$ of our hypothetical mechanism. Accordingly, the function, $\log \frac{\sqrt{i}}{1-\sqrt{i}}$, in which i is the fractional short-circuit current, was computed and plotted *vs.* pH. The result is seen in Fig. 5. Provided the dissociation constants are of equal magnitude, the slope of this function *vs.* pH should be unity, if $n = 2$. In Fig. 5 the solid line is drawn with unit slope, and as may be seen, the data scatter rather uniformly about it. Furthermore, the pH at which the function, $\log \frac{\sqrt{i}}{1-\sqrt{i}} = 0$, represents the pH of the dissociation reactions and is seen to be about 6.7–6.8. The value we have employed to gain best fit to our experimental data is 6.9, and thus there is again good agreement.

The curve generated by Eqn. (6) is also very sensitive to the values of the two rate constants, k_c and k_b, employed. This is especially so with respect to the relative position of the maximum short-circuit current in the time following the step change in conditions. As has been stated, the rate constants are related to the diffusion coefficients and the thickness of the skin. Assuming a skin thickness of 0.04 cm, a value obtained by WRIGHT[8], a k_c of 0.36 min^{-1}, the value used to obtain the solid curves of Fig. 4 above, corresponds to a diffusion coefficient for CO_2 of $5.7 \cdot 10^{-4}$ $cm^2 min^{-1}$. This is just the value that WRIGHT[8] has given for the diffusion of CO_2 through frog skin. Since, however, the thickness of the skin enters as the inverse square, and there is the uncertainty as to whether to consider the skin a symmetrical structure with respect to CO_2 diffusion, the agreement of our value with that of WRIGHT, obtained by other methods, is perhaps somewhat fortuitous. The rate constant, k_b, of bicarbonate ion corresponds to a diffusion coefficient of $1.6 \cdot 10^{-4}$ $cm^2 min^{-1}$ utilizing the same value as above for a skin thickness. The diffusion coefficients of common univalent electrolytes in aqueous solution is of the order of $1.0-1.2 \cdot 10^{-4}$ $cm^2 min^{-1}$.[9]

Although there exists a rational physical basis for the parameters used in the analysis of the hypothetical mechanism, and a definite reasonableness of their values in obtaining a fit to the experimental data, it is to be emphasized that no claim is made for the details of the model assumed. The analysis is based upon the simplest model conceived which yet serves adequately to portray the data. Suffice it to say that the effect of CO_2 on the sodium transport system of isolated frog skin is entirely explicable in terms of an effect of a concomitant and dependent hydrogen ion concentration. The observed differences in effect of CO_2–HCO_3^- buffer systems and phosphate buffer systems are then accounted for in terms of the relatively rapid diffusion of CO_2 leading to local changes in pH at the site of sodium transport.

ACKNOWLEDGEMENT

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STUDIES ON THE MECHANISM OF PROTEIN SYNTHESIS; INCORPORATION OF *p*-FLUOROPHENYLALANINE INTO *α*-AMYLASE OF *BACILLUS SUBTILIS*

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SUMMARY

1. Cell growth of *Bacillus subtilis* and appearance of α-amylase activity in the culture medium are inhibited by *p*-fluorophenylalanine.

2. *p*-Fluorophenylalanine is incorporated into the α-amylase molecule to the same extent as in the total cellular protein.

3. Complete amino acid analysis showed that some of the phenylalanine residues in α-amylase molecule are replaced by *p*-fluorphenylalanine.

4. α-Amylase containing *p*-fluorphenylalanine showed the same sedimentation velocity, viscosity and electrophoretical behavior as the normal enzyme, but it had reduced enzymic activity.

5. The amino acid composition of α-amylase was determined.

INTRODUCTION

Interesting findings concerning the problem of amino acid analogue incorporation into proteins are accumulating. Cowie *et al.* reported that the protein pattern in bacterial cells is not radically altered by the incorporation of amino acid analogues[1].

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Previous papers from our laboratory[2,3], on *Bacillus subtilis* α-amylase, showed that ethionine can be incorporated into the normal amino acid sequence of the α-amylase molecule uniformly replacing methionine, and that the enzyme containing ethionine has the same physico-chemical properties and enzymic activity as the normal enzyme.

It has also been observed that some amino acid analogues inhibit cell growth as well as enzyme formation[4,5]. However, in the case of ethionine, the enzyme containing it has just the same activity as the normal enzyme[2]. Nevertheless it might be argued that the enzyme containing other amino acid analogues may be less active.

In the present work, using *p*-fluorophenylalanine as an amino acid analogue, it was observed that cell growth and α-amylase formation were suppressed by the amino acid analogue. However, the α-amylase molecule containing *p*-fluorophenylalanine had the same physico-chemical properties as the normal enzyme, but it was less active.

MATERIALS AND METHODS

Bacterial strain

Bacillus subtilis strain K obtained from the Institute of Applied Microbiology of the University of Tokyo was used in these experiments.

Materials used

DL-*p*-Fluorophenylalanine was synthesized by OKUDA AND TATSUMI at the Protein Institute of the University of Osaka[6]. The other materials used were the same as described in a previous paper[3].

Culture method

The bacterial strain was grown on the surface of the synthetic basal medium[3] or the basal medium containing DL-*p*-fluorophenylalanine at 37°.

Isolation and crystallization of α-amylase

α-Amylase was prepared from the culture medium by the procedures described previously[3]. It was lyophylized after having been recrystallized at least three times.

Assay of α-amylase activity

The enzyme activity was measured by the method described previously[3].

Measurements of sedimentation and viscosity

Ultracentrifugation experiments were carried out in a Spinco Model-E centrifuge at a speed of 59,780 rev./min at room temperature. The concentration of the protein was 0.9 %, in acetate buffer, pH 6.0 and 0.2 ionic strength.

The viscosity of 0.4 to 0.9 % protein in the same buffer, was measured in an Ostwald viscosimeter at 20°.

Paper electrophoresis

Paper electrophoresis was carried out in 0.1 *M* Tris buffer at pH 8.2 and 8.8 at 0.8 mA/cm in a cold room for 20 h. Whatman No. 1 filter paper was used.

Quantitative estimation of amino acids

α-Amylase was hydrolysed completely in redistilled HCl at 112–113° for 24 h. The hydrolysates were evaporated to dryness in a vacuum over NaOH.

In the case of total cellular protein, acetone-dried cells were washed twice with 10 % trichloracetic acid, and three times with ethanol. After drying, the powder was hydrolysed as described above.

Chromatographic analysis was performed on Amberlite IR-120, using a 0.9×150 cm column for the acidic and neutral amino acids, and a 0.9×15 cm column for the basic amino acid and ammonia, as described by MOORE, SPACKMAN AND STEIN[7]. The amino acids were determined by the ninhydrin method[8]. For the estimation of proline by the ninhydrin method the extinction at 440 mμ was measured; Chinard's method[9] was also used. Tryptophan was estimated by measuring the extinction at 294.4 and 280 mμ correcting for spurious absorption as described by GOODWIN AND MORTON[10].

The ratio between *p*-fluorophenylalanine content and phenylalanine content in hydrolysates of α-amylase and cellular protein was estimated as follows. After elimination of the aliphatic amino acids by charcoal treatment[11], the two amino acids were estimated by quantitative paper chromatography[12] using Whatman No. 1 and *tert*-butanol–methyl ethyl ketone–water (4:4:1.5). Small amounts of leucine and isoleucine, which were not eliminated by the charcoal treatment, were present in the phenylalanine spot. The amounts were estimated by paper chromatography using Whatman No. 1 and *n*-butanol–formic acid–water (75:20:5), and subtracted for correction.

RESULTS

Effect of p-fluorophenylalanine on bacterial growth and α-amylase formation.

The relations between cell growth, development of α-amylase activity and concentration of *p*-fluorophenylalanine in the culture medium are shown in Fig. 1 and Fig. 2. It will be seen that bacterial growth is reduced by *p*-fluorophenylalanine and the rate of appearance of α-amylase activity is still more reduced. It has been observed

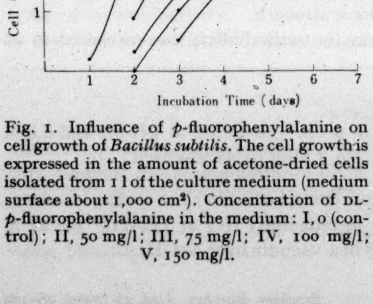

Fig. 1. Influence of *p*-fluorophenylalanine on cell growth of *Bacillus subtilis*. The cell growth is expressed in the amount of acetone-dried cells isolated from 1 l of the culture medium (medium surface about 1,000 cm^2). Concentration of DL-*p*-fluorophenylalanine in the medium: I, 0 (control); II, 50 mg/l; III, 75 mg/l; IV, 100 mg/l; V, 150 mg/l.

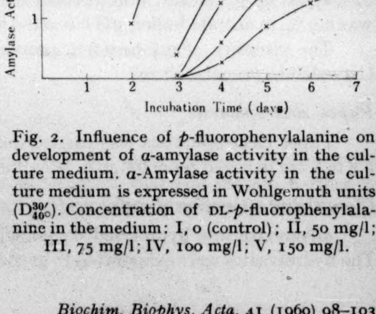

Fig. 2. Influence of *p*-fluorophenylalanine on development of α-amylase activity in the culture medium. α-Amylase activity in the culture medium is expressed in Wohlgemuth units ($D^{30'}_{40°}$). Concentration of DL-*p*-fluorophenylalanine in the medium: I, 0 (control); II, 50 mg/l; III, 75 mg/l; IV, 100 mg/l; V, 150 mg/l.

that in the presence of certain amino acid analogues cell growth becomes linear, not logarithmic[4], with time. Our results shown in Fig. 1 are in accordance with these observations.

Amino acid composition of α-amylase and p-*fluorophenylalanine content of α-amylase and total cellular protein*

The amino acid composition of normal α-amylase and α-amylase isolated from the culture medium containing 72 mg/l of DL-*p*-fluorophenylalanine, is presented in Table I. These values are the means of duplicate analyses. It will be seen that the amino acid composition of α-amylase containing *p*-fluorophenylalanine and that of the normal enzyme are identical within the experimental error, with the exception of the phenylalanine content. This leads to the conclusion that 9 % of the phenylalanine in α-amylase molecule was replaced by *p*-fluorophenylalanine.

TABLE I

AMINO ACID COMPOSITION OF NORMAL α-AMYLASE AND α-AMYLASE CONTAINING *p*-FLUOROPHENYLALANINE

Amino acid	*Amino acid g/100 g of protein*		*Assumed No. of residues/molecule* §
	Normal α-amylase	*α-Amylase conaining* p-*Fluotophenylalanine*	
Aspartic acid	13.8 ± 0.8	14.7 ± 1.1	53
Threonine*	5.46 ± 0.15	5.75 ± 0.06	23
Serine**	4.95 ± 0.08	5.05 ± 0.15	23
Glutamic acid	12.80 ± 0.62	13.05 ± 0.80	43
Proline	3.40 ± 0.06	3.29 ± 0.00	14
Glycine	5.52 ± 0.06	5.68 ± 0.05	37
Alanine	4.98 ± 0.12	5.34 ± 0.12	28
Valine	5.93 ± 0.18	5.99 ± 0.00	25
Methionine	1.45 ± 0.25	1.41 ± 0.03	5
Isoleucine	4.38 ± 0.20	4.72 ± 0.23	17
Leucine	6.15 ± 0.23	6.40 ± 0.07	23
Tyrosine	8.40 ± 0.17	8.63 ± 0.30	23
Phenylalanine	6.10 ± 0.05	5.46 ± 0.20	18
p-Fluorophenylalanine		0.49 ± 0.02	
Lysine	7.98	7.75 ± 0.03	26
Histidine	3.62	3.44 ± 0.17	11
Arginine	5.89	6.07 ± 0.20	17
Tryptophan	5.95	6.27	15
Ammonia***	1.78	1.96 ± 0.05	
Total	106.76	109.29	401

The values are the means of duplicate determinations.

* The recovery is assumed to be 92 % after 24 h hydrolysis according to JUNGE *et al.*[15].

** The recovery is assumed to be 80 % after 24 h hydrolysis according to JUNGE *et al.*[15].

*** Not included in the total.

§ The molecular weight is assumed to be 49,000 according to JUNGE *et al.*[15].

The ratio of *p*-fluorophenylalanine content and phenylalanine content in the α-amylase molecule and in the cellular protein isolated from the culture medium containing 72 or 150 mg *p*-fluorophenylalanine per l is shown in Table II. The results, which were obtained by quantitative paper chromatography, indicate that phenylalanine has an equal chance of being replaced by *p*-fluorophenylalanine in the α-amylase molecule and in the cellular protein, and that the degree of replacement is related to the concentration of *p*-fluorophenylalanine in the medium.

Enzymic activity of α-amylase containing p-*fluorophenylalanine*

The enzymic activity of α-amylase containing *p*-fluorophenylalanine, expressed in percentages of the activity of the normal enzyme, is shown in Table II. The fluctuation in enzyme activity of various enzyme preparations was not greater than ± 10 %. It will be seen that α-amylase containing *p*-fluorophenylalanine has reduced enzymic activity, while the enzyme containing the highest amount of the amino acid analogue shows the greatest decrease of activity.

TABLE II

p-FLUOROPHENYLALANINE CONTENT IN α-AMYLASE AND CELLULAR PROTEIN AND ENZYME ACTIVITY OF α-AMYLASE CONTAINING *p*-FLUOROPHENYLALANINE

	p-*FPHE/PHE*	*Enzyme activity*
α-Amylase separated from the medium containing 72 mg/l of *p*-FPHE	0.08 ± 0.02 (0.09)*	85 ± 7
α-Amylase separated from the medium containing 150 mg/l of *p*-FPHE	0.15 ± 0.02	70 ± 7
Cellular protein separated from the medium containing 72 mg/l of *p*-FPHE	0.08 ± 0.02	
Cellular protein separated from the medium containing 150 mg/l of *p*-FPHE	0.13 ± 0.02	

The ratio of *p*-fluorophenylalanine content to phenylalanine content was determined by quantitative paper chromatography. The enzyme activities are expressed in percentage of the activity of normal α-amylase. Abbreviations: PHE, phenylalanine; *p*-FPHE, *p*-fluorophenylalanine.

* Calculated from figures in Table I.

Physico-chemical properties of α-amylase containing p-*fluorophenylalanine*

Schlieren patterns of α-amylase *p*-fluorophenylalanine (*p*-fluorophenylalanine/phenylalanine = 0.09) showed one symmetrical peak, indicating a high degree of homogeneity. The sedimentation constant ($S_{20,w}$) was 4.19 S, a value identical with that of normal α-amylase (4.22 *S*)[3].

The intrinsic viscosity of α-amylase containing *p*-fluorphenylalanine (*p*-fluorophenylalanine/phenylalanine = 0.09 and 0.15) was 5.1 ± 0.2 ml/g, thus within experimental error also identical with that of the normal enzyme (5.4 ± 0.1 ml/g).

Patterns of paper electrophoresis at pH 8.2 and 8.8 of α-amylase containing *p*-fluorophenylalanine (*p*-fluorophenylalanine/phenylalanine = 0.09 and 0.15) indicated the same electropheretical mobility as that of normal enzyme as well as the homogeneity of the preparations.

DISCUSSION

Complete amino acid analysis shows that the amino acid composition of α-amylase isolated from the culture medium containing *p*-fluorophenylalanine is identical with that of normal α-amylase, with the exception of the phenylalanine content.

Kruh and Rosa[13] observed that *p*-fluorophenylalanine incorporation into hemoglobin is competitively inhibited by phenylalanine, but not by tyrosine; accordingly it was presumed that *p*-fluorophenylalanine can only replace phenylalanine. Our results suggest that this also holds for the incorporation of *p*-fluorophenylalanine in α-amylase.

In a previous paper[3], it has been established that ethionine can be incorporated into protein by "uniformly" replacing methionine. This suggests that all phenylalanine groups in the protein may also have an equal chance of being replaced by *p*-fluorophenylalanine.

The amino acid composition of α-amylase produced by *Bacillus subtilis* has been reported by Akabori *et al.*[14] and by Junge *et al.*[15]. The values obtained in the present analysis are in good agreement with their results, especially with those of Junge *et al.*

In spite of the fact that no difference could be detected between the sedimentation velocity, viscosity and electrophoretical behavior of α-amylase containing *p*-fluorophenylalanine and those of normal enzyme, the former showed reduced enzymic activity. It may be that one or a few phenylalanine residues in the α-amylase molecule are intimately related with its activity, and that *p*-fluorophenylalanine incorporation into such specific phenylalanine residues leads to inactivation of the enzyme. An alternative hypothesis is that α-amylase is partially inactivated by replacement of any of the phenylalanine residues in the molecule. In this connection, it will be interesting to study the incorporation of an analogue of an amino acid that is considered to be a group essential for enzyme activity.

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PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM

III. DETERMINATION OF NEW PEPTIDE BONDS; REQUIREMENT FOR THE "AMINO ACID INCORPORATION ENZYME" IN PROTEIN BIOSYNTHESIS

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SUMMARY

The incorporation of [^{14}C] amino acids into protein by *Alcaligenes faecalis* fragments has been demonstrated. Using [^{14}C]valine, it was found that most of the incorporated amino acid was within the peptide chains. *Alcaligenes* fragments have been partially solubilized by treatment with perfluoro octanoate without affecting the incorporation activity. The soluble fraction obtained after this treatment can be replaced by purified "amino acid incorporation enzymes".

INTRODUCTION

In two previous papers BELJANSKI AND OCHOA have reported that a particulate preparation of *Alcaligenes faecalis* is able to incorporate various [^{14}C] amino acids into protein[1,2]. It was also shown that under appropriate conditions, a highly purified enzymic preparation obtained from crude extracts of this organism stimulates the incorporation of amino acids when added to a particulate preparation. The purified enzyme preparation had no ability to promote the exchange of labeled pyrophosphate with ATP in the presence of amino acids, yet it completely replaced the "pH 5.0 enzymes" in the incorporation of amino acids into protein of rat liver microsomes[2]. This new enzymic preparation was referred to as the "amino acid incorporation enzyme".

In this paper, evidence is presented to show that the incorporated [^{14}C] amino acids are in true peptide bonds. We further wish to report results indicating that the same particulate preparation appears to be free of so called "amino acid activating enzymes" and contains the "amino acid incorporation enzyme". This latter system is able to catalyse the Mg^{++} dependent exchange of four labeled diphosphonucleosides

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Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; PP, pyrophosphate; Tris, tris (hydroxymethyl) aminomethane; FDNB, 2,4-fluoro-dinitrobenzene.

with their homologous triphosphonucleosides[3]. The exchange reactions seem to be related to the incorporation of amino acids into peptide bond.

MATERIALS AND METHODS

The preparation of *Alcaligenes faecalis* (8750) fragments and the determination of the incorporation of $[^{14}C]$ amino acids into protein have been described previously[1].

Solubilisation of Alcaligenes *particles*

Partial solubilisation of the particulate preparation has now been achieved with sodium perfluoro-octanoate (Minnesota Mining and Manufacturing Co., St Paul 6, Minn., U.S.A.). 1 ml of the particulate preparation in Tris buffer, 0.05 *M*, pH 7.3, containing 10–15 mg of protein was mixed with 2 mg of perfluoro-octanoate dissolved in 0.1 ml of distilled water. After standing at room temperature for 25 min, the lysed preparation (L) was centrifuged at 30,000 × *g* for 25 min in the cold giving a pellet (P 30) and a yellow-brown supernatant (S 30) which contained about 50 % of the initial protein (ratio 280/260 = 1.7). The pellet (P 30) was resuspended in the same volume of Tris buffer as the original mixture (ratio 280/260 = 1.0).

Peptide bond determination

In order to show the position of the labeled amino acid in the newly formed protein, a large amount of labeled protein was obtained by incubation of the particulate preparation containing 20 mg of protein with uniformally labeled $[^{14}C]$valine (0.04 *µM*, 500,000 counts/min) and a mixture of 18 L-amino acids as previously described[1].

Effect of carboxypeptidase: The incorporation of $[^{14}C]$valine into the C-terminal position of peptide chains was tested with crystalline carboxypeptidase[4] (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.). The conditions of incubation are described in the legend to Table IV. After stopping the reaction with 5 ml of 10 % trichloroacetic acid (TCA), the precipitated protein was collected by centrifugation, washed twice with cold TCA, dried with acetone and ether and the radioactivity measured. Any decrease in radioactivity of the protein after treatment with carboxypeptidase would indicate the occurrence of incorporated $[^{14}C]$valine in the C-terminal position of peptide chains. Carboxypeptidase activity was tested beforehand with native and denatured bovine serum albumin. Protein was measured by the biuret reaction.

Treatment with fluoro-dinitrobenzene: The possible presence of $[^{14}C]$valine in the N-terminal position of peptide chains was tested with 2,4-fluoro-dinitrobenzene (FDNB)[5]. 10 mg of protein labeled with $[^{14}C]$valine (1300 counts/min/mg) were mixed with 10 mg of $NaHCO_3$ in 1 ml of water and 2 ml of an ethanolic solution of FDNB, (5 % v/v). The mixture was vigorously shaken at room temperature for 2 h. A few drops of concentrated HCl were then added and the protein was washed several times with ether. The protein was finally hydrolysed with 6 *N* HCl for 16 h at 110°. The DNP-amino acids were separated from free amino acids by extraction with ether. The radioactivity of dried samples of the ether extract and the water solution was determined.

Partial hydrolysis of labeled protein: The incorporation of $[^{14}C]$valine within peptide chains was demonstrated by partial hydrolysis of the protein with HCl and isolation and analysis of labeled peptides. 20 mg of labeled protein (1360 counts/min/mg) were dissolved in 1 ml of concentrated HCl and allowed to stand for 4 days at 37°. The HCl was removed by evaporation in the presence of distilled water. Aliquots of the hydrolysate were chromatographed on Whatman No. 2 paper in two dimensions. The separated peptides were detected by radioautograph. Three of the distinct radioactive materials resulting from the partial hydrolysis of the protein were eluted and were each hydrolysed with 6 *N* HCl for 24 h at 110°. After removal of HCl by evaporation each sample was chromatographed. The chromatogram was sprayed with ninhydrin and then exposed to film.

ATP–PP exchange: The exchange of ATP with [^{32}P]pyrophosphate was measured as previously described[1].

Exchange of labeled di- with unlabeled triphosphonucleosides: The method for measurement of exchange of labeled diphosphonucleosides with unlabeled triphosphonucleosides is described in the following paper[6]. All unlabeled di- and triphosphonucleosides were purchased from Sigma Chemical Company (St Louis Ill., U.S.A.).

Myokinase activity: Myokinase activity of the particulate preparation of *Alcaligenes faecalis* was tested by incubation of [^{14}C]ADP with Mg^{++} ions and subsequent analysis for appearance of labeled ATP and AMP. Essentially no myokinase activity was detected.

RESULTS

Amino acid incorporation into protein by particulate preparation of Alcaligenes faecalis

A number of [^{14}C] labeled amino acids were tested for their ability to be incorporated into protein by *Alcaligenes faecalis* particulate preparations. As shown in Table I all of the L- or DL-amino acids tested were incorporated into protein. It is interesting to note that the addition of unlabeled D-leucine, and D-valine did not depress the incorporation of their respective labeled L-enantiomorphs; on the other hand addition

TABLE I

INCORPORATION OF ^{14}C LABELED AMINO ACIDS INTO PROTEIN BY *Alcaligenes faecalis* PARTICULATE PREPARATION

The samples contained: Tris buffer, pH 7.3, 50 µmoles; $MgCl_2$, 5.0 µmoles; K_2HPO_4, 5.0 µmoles; 0.2 µmole of labeled (120,000 counts/min/µmole) and unlabeled amino acid; unwashed *Alcaligenes* particles with 300–400 µg of protein[1]. Final volume, 1.0 ml; incubation in air, 1 h at 34°. Addition of ATP had little stimulating effect. The system seems to depend on oxidative phosphorylation for generation of the ATP[1].

Added amino acids		*Labeled amino acids incorporated mµ M/mg protein N/h*
Labeled	*Unlabeled*	
Glycine (0 time)		0
Glycine		44
L-valine		18
L-valine	D-valine	18
DL-leucine		24
DL-leucine	D-leucine	24
DL-phenylalanine		26
DL-phenylalanine	D-phenylalanine	19
DL-glutamic acid		12
DL-glutamic acid	D-glutamic acid	5

of D-phenylalanine and D-glutamic acid did depress the incorporation of the labeled amino acids (Table I). Similar results have been obtained with perfluoro-octanoate treated preparations.

Chloramphenicol effect: Since chloramphenicol is generally considered to be an inhibitor of protein biosynthesis[7-9] but not of cell wall synthesis in bacteria[10], the effect of this antibiotic in incorporation of labeled amino acids into protein by the *Alcaligenes faecalis* particulate preparation was tested. Addition of chloramphenicol at different concentrations to the incubation mixture without preincubation of particles with antibiotic resulted in strong inhibition of amino acid incorporation, Table III.

Peptide bond formation: Previous observations have suggested strongly the formation of true peptide bonds when amino acids are incorporated into hot TCA insoluble material by *Alcaligenes faecalis* particles[1,2]. In the present studies the labeled protein obtained after incubation with [^{14}C]valine was treated with carboxypeptidase and with FDNB and was also partially hydrolysed with hydrochloric acid to obtain labeled peptides. Treatment of the labeled protein with carboxypeptidase

TABLE II

INCORPORATION OF [^{14}C]GLYCINE INTO PROTEIN OF *Alcaligenes faecalis* PARTICULATE PREPARATION UNTREATED AND TREATED WITH PERFLUORO-OCTANOATE

Conditions as in Table I: with 300 μg of protein of washed particles[1]; supernatant (S 30) with 150 μg of protein; pellet (P 30) with 150 μg of protein; "amino acid incorporation enzyme" (specific activity 20) 20 μg of protein.

Preparation	*[^{14}C]glycine incorporated mμ M/mg of protein N/h*
Untreated (0 time)	0
Untreated	20
Treated (L)	25
Pellet (P 30)	4
Supernatant (S 30)	0
Pellet (P 30) + supernatant (S 30)	30
Pellet (P 30) + heated supernatant (S 30)	10
Pellet (P 30) + "amino acid incorporation enzyme"	26

TABLE III

EFFECT OF CHLORAMPHENICOL ON INCORPORATION OF [^{14}C] AMINO ACIDS INTO PROTEIN OF *Alcaligenes faecalis* PARTICULATE PREPARATION

Conditions as in Table I: unwashed *Alcaligenes* particles[1] with 350 μg of protein; chloramphenicol 50 μg and 100 μg/ml.

Added labeled amino acids	*Labeled amino acid incorporated mμ M/mg of protein N/h*		
	Chloramphenicol		
	No addition	*Addition*	
		50 μg	*100 μg*
Glycine	55	8	5
DL-leucine	13	1.5	0.4
DL-glutamic acid	13	2.5	2.1

had no significant effect on the radioactivity of the TCA insoluble material (Table IV). Although this is not absolute proof, the negative effect obtained with carboxypeptidase suggests that [^{14}C]valine was not present in any significant quantity in the C-terminal position of peptide chains.

Table V shows that when the N-terminal position of the incorporated amino acid was determined with the DPN-method[5] about 15 % of the [^{14}C]valine reappeared as DNP-valine. This indicates that although the labeled amino acid was incorporated primarily within the peptide chains, a significant quantity of the valine was present at the N-terminal position.

TABLE IV

RADIOACTIVITY OF LABELED PROTEIN WITH [^{14}C]VALINE OF *Alcaligenes* PARTICLES TREATED WITH CARBOXYPEPTIDASE

The samples contained: phosphate buffer, pH 7.4, 0.5 *M*; 7.6 mg of [^{14}C]protein (1350 counts/min/mg); 1350 µg of carboxypeptidase in 0.03 ml of 13 % LiCl; final volume 0.64 ml; Temperature, 38°.

Incubation time (hours)	*Counts/min*	*Protein mg*	*Counts/min/mg protein*
0	1400	1.05	1350
1	1646	1.25	1320
7	1230	1.00	1230

TABLE V

N-TERMINAL POSITION OF [^{14}C]VALINE IN PROTEIN OF *Alcaligenes* PARTICULATE PREPARATION

Conditions as described in MATERIALS AND METHODS.

Preparation	*Total counts/min*
Labeled protein with [^{14}C]valine	13,500
DNP [^{14}C] amino acid	2,000
Free [^{14}C] amino acid	11,550

Partial hydrolysis with HCl and chromatography of the hydrolysate revealed the existence of a number of radioactive residues (Fig. 1). After elution of the spot corresponding to position "A", hydrolysis and chromatography of the eluted material revealed the existence of six amino acids by the ninhydrin test, only one of which was radioactive on exposure to X ray film. The ninhydrin positive, radioactive spot corresponded to that expected for valine (Fig. 2). This procedure was also carried out

Isolation of peptides labeled with [^{14}C]valine from partially hydrolysed protein of *Alcaligenes* particles.

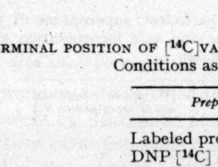

Fig. 1. Chromatogram. Peptide spots printed on X ray film.

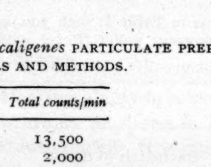

Fig. 2. Chromatogram. Amino acids from hydrolysed spot "A" from Fig. 1.

Solvent 1, *n*-butanol–acetic acid–water (250:60:250); solvent 2, phenol–water (4:1).

with similar results with two other labeled peptides. These observations demonstrate that [^{14}C]valine was to a great extent incorporated into peptide bonds within the peptide chains.

Dependency of incorporation of amino acids into Alcaligenes *fragments on the content of the "amino acid incorporation enzyme"*

Previous results have shown that a particulate preparation of *Alcaligenes faecalis* incorporates ^{14}C amino acids into protein. However, we were unable to detect the presence of "amino acid activating enzymes" in this preparation[1]. In the present study pretreatment with perfluoro-octanoate was used to partially solubilise the particulate preparation. As shown in Table VI the treated preparation does not show any detectable pyrophosphate–ATP exchange activity. The supernatant obtained at 30,000 × *g* of the treated preparation contains the "amino acid incorporation enzyme" activity. This fraction also is capable of catalysing a rapid exchange of all four di- with triphosphonucleosides (Table VI).

TABLE VI

PRESENCE OF THE "AMINO ACID INCORPORATION ENZYME" IN THE *Alcaligenes faecalis* PARTICULATE PREPARATION UNTREATED AND TREATED WITH PERFLUORO-OCTANOATE

Conditions as described in the following paper[6]: with 220 μg of protein of untreated and treated (L) *Alcaligenes faecalis* particulate preparation (washed particles[1]; 115 μg of supernatant (S 30) and 100 μg of pellet (P 30)). Incubation time 30 min. Calculations according to DE MOSS AND NOVELLI[22].

Preparation	*Rate of exchange; μmoles/mg protein/h*				
	ATP–ADP	*GTP–GDP*	*UTP–UDP*	*CTP–CDP*	*ATP–^{32}P^{32}P*
Untreated	0.75	0.85	0.90	0.60	0.00
Treated (L)	1.90	1.75	1.65	1.14	0.00
Supernatant (S 30)	3.20	3.40	2.95	2.05	0.00
Pellet (P 30)	0.40	0.70	0.75	0.60	0.00

When the supernatant solution was used under the conditions described in the legend to Table II, no [^{14}C] amino acids were incorporated into protein. The pellet (P 30) obtained after perfluoro-octanoate treatment still retained some incorporation activity. Combination of the two fractions resulted in an incorporation into protein which was equal to the incorporation of amino acids exhibited by the *Alcaligenes faecalis* preparation treated with perfluoro-octanoate without centrifugation.

It was found that the addition of purified "amino acid incorporation enzyme" to the pellet (P 30) instead of the supernatant fraction (S 30) resulted in a stimulation of amino acid incorporation into protein which is equivalent to that obtained with untreated fragments (Table II). This purified fraction which completely replaced the supernatant solution contains no amino acid activating enzymes[1].

When the 40 % ammonium sulfate fraction[1] which is a fraction rich in amino acid activating enzymes was added to the perfluoro-octanoate pellet (P 30) in the presence of ATP no stimulation of the incorporation of amino acids was observed.

DISCUSSION

In our previous paper we have described a particulate preparation of *Alcaligenes faecalis* which is capable of active incorporation of amino acids into protein although the amino acid dependent exchange of PP with ATP was not detectable[1]. In further study of this same material presented here, it is more definitely shown that the "amino acid activating enzymes" are absent in the system, since the perfluoro-octanoate solubilized particles do not show, in detectable manner, the ability to activate the amino acids by ATP. This is particularly interesting in view of the suggestion by Hoagland and his colleagues[11] and others[12–15] that the carboxyl activation of amino acids is the first step in protein biosynthesis. On the other hand, the same partially solubilised material is able to incorporate [^{14}C] amino acids into peptide bonds. Similar observations were made by Cohn[18] and Zalta[19] with microsomal systems from animal tissues. Recently, Nisman and Fukuhara have described the properties of a "soluble fraction" called "amino acid polymerase" isolated from cell membrane fragments of *E. coli*. This preparation actively incorporates amino acids into protein in the absence of amino acid activating enzymes[20]. The above results suggest that the incorporation of amino acids into protein does not necessarely involve preliminary formation of amino acyl adenylates.

It is particularly interesting to note that the "amino acid incorporation enzyme" which is able to replace the requirement for the solubilized fraction (S 30) of *Alcaligenes* fragments shows the ability to exchange phosphate between all four di- and triphosphonucleosides. This suggests that the latter activity may be directly or indirectly involved in protein biosynthesis. It is also of interest to recall the experiments of Snoke and Bloch[21] on glutathione formation where it was shown that a highly purified enzyme which catalysed the formation of glutathione, also catalysed the exchange of orthophosphate from ATP to ADP.

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PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM

IV. EXCHANGE OF DIPHOSPHONUCLEOSIDES WITH HOMOLOGOUS TRIPHOSPHONUCLEOSIDES BY THE "AMINO ACID INCORPORATION ENZYME"

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SUMMARY

A highly purified preparation of the "amino acid incorporation enzyme" is capable of catalysing the transfer of phosphate from triphosphonucleosides to homologous diphosphonucleosides (ATP–ADP; UTP–UDP; CTP–CDP; GTP–GDP). Thermal inactivation of this enzyme preparation suggests the existence of four different and specific enzymes each capable of catalysing the Mg^{++} dependent exchange of one pair of nucleotides. The purified enzymic preparation is free of diphosphonucleoside kinase (ATP + UDP $\rightleftharpoons$ UTP + ADP), monophosphonucleoside kinase (ATP + UMP $\rightleftharpoons$ ADP + UDP) and myokinase (2 ADP $\rightleftharpoons$ ATP + AMP).

INTRODUCTION

We have shown that a purified fraction obtained from *Alcaligenes faecalis* promotes the incorporation of amino acids into peptide bonds when added to a particulate fraction[1,2]. The purified fraction which we have called the "amino acid incorporation enzyme" also catalyses the exchange reaction of [^{14}C]ADP with ATP.

In this paper we present data with respect to the ability of this enzymic preparation to catalyse the exchange of all four di- with triphosphonucleosides and have attempted to determine whether these activities were due to a single or to several enzymes. A brief summary of these results has been presented in a preliminary note[3].

MATERIALS AND METHODS

Enzyme purification

The method of purification of the amino acid incorporation enzyme already described[1] has been slightly modified. In step 2, the ammonium sulfate concentration was increased to 0.70%. It was found that in step 5 the use of an ammonium sulfate

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Abreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; PP, pyrophosphate; Tris, tris(hydroxymethyl)aminomethane.

TABLE I

EXCHANGE OF DI- WITH TRIPHOSPHONUCLEOSIDES BY THE "AMINO ACID INCORPORATION ENZYME"

Incubation mixture: 5 μM of $MgCl_2$; 50 μM of Tris, pH 7.3; 0.5 μM of each nucleotide; 3 μg of purified "amino acid incorporation enzyme". Final volume 0.5 ml. Incubation 10 min in air at 34°. Blank values representing from 3 to 5 % of exchange have been substracted. Calculations according to DE MOSS AND NOVELLI[9].

System	*Time of incubation (min)*	*Radioactivity, counts/min*								*Exchange per cent of final equilibrium*	*Rate of exchang μmoles/mg protein/h*
		ADP–ATP		*GDP–GTP*		*UDP–UTP*		*CDP–CTP*			
Complete	0	2,506	0	15,900	0	16,080	0	11,820	0	0	0
Complete	10	1,670	770							62	486
Complete	10			10,810	4,808					61	483
Complete	10					10,700	5,280			65	530
Complete	10							7,800	3,800	58	430
No $MgCl_2$	10	2,460								0	0
No enzyme	10	2,470								0	0
No ATP	10	2,458								0	0
Heated enzyme	10	2,465								0	0

concentration of 0.40 % instead of 0.55 % resulted in a better yield of the "amino acid incorporation enzyme" while still allowing a clear cut separation of this enzyme from the "amino acid activating enzymes" fraction. The former enzyme was then precipitated from the supernatant solution by 0.70 % ammonium sulfate, dissolved in a minimum volume of phosphate buffer, 0.01 M, pH 7.4, and dialysed against 200 volumes of the same buffer at 3° for 1 h with one change of dialysing solution.

ATP–PP exchange. The method of assay of the exchange between ATP and ^{32}P labeled pyrophosphate has been described[1].

Diphosphonucleosides–triphosphonucleosides exchange

The conditions of measurement of the exchange of labeled diphosphonucleosides with unlabeled triphosphonucleosides are described in the legend to Table I. The reaction was stopped with 0.05 ml of 40% TCA. The nucleotides were separated by chromatography on Whatman No. 1 paper[4] and the u.v. absorbing spots detected. After elution the radioactivity of nucleotides was measured. [8-^{14}C]ADP was purchased from the Schwartz Lab. (Mount Vernon, New York). GDP, UDP and CDP were labeled in the terminal phosphate with ^{32}P using a polynucleotide phosphorylase preparation from *Alcaligenes faecalis* (S.A. 4) according to the method of GRUNBERG-MANAGO AND OCHOA[5]. The labeled diphosphonucleosides were twice chromatographed on Whatman 3 MM paper, eluted with water and lyophylised. All unlabeled di- and triphosphonucleosides were purchased from Sigma Company (St Louis Ill. U.S.A.).

Diphosphonucleoside kinase activity

The presence of diphosphonucleoside kinase in the purified "amino acid incorporation enzyme" was tested by incubation of [^{14}C]ADP with either CTP, GTP or UTP and subsequent analysis for the appearance of labeled ATP[6]. The incubation conditions are described in the legend to Table II. After stopping the reaction with 0.05 ml of 40% TCA, the nucleotides were chromatographed and eluted as described for the exchange of diphosphonucleosides with their homologous triphosphonucleosides.

TABLE II

ASSAY OF NUCLEOSIDE DIPHOSPHOKINASE ACTIVITY[6] IN THE PURIFIED "AMINO ACID INCORPORATION ENZYME"

Conditions of incubation: 5 μM of $MgCl_2$; 50 μM of Tris buffer pH 7.3; 1 μM of each nucleotide ([^{14}C]ADP with 2,300 counts/min); 3 μg of purified "amino acid incorporation enzyme". Final volume 0.5 ml. Incubation 10 min in air at 34°. Nucleotides were separated as described in MATEPIALS AND METHODS.

Labeled substrate	*Addition*	*counts/min found in ATP*
[^{14}C]ADP	ATP	1,000
[^{14}C]ADP	GTP	0
[^{14}C]ADP	UTP	0
[^{14}C]ADP	CTP	0

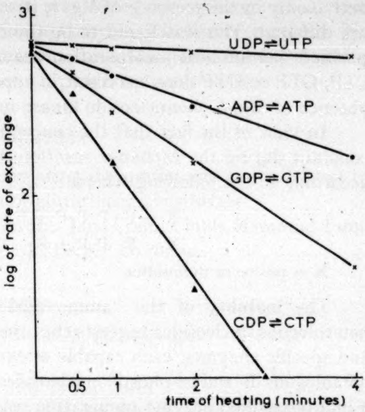

Fig. 1.

Myokinase activity. The myokinase activity was tested as previously described[3]. No activity was detected.

RESULTS AND DISCUSSION

In previous experiments it was observed that certain preparations of the "amino acid incorporation enzyme" which catalysed a very rapid exchange of [^{14}C]ADP with ATP, failed to stimulate the incorporation of amino acids into *Alcaligenes* particles. These preparations which were active only for ADP–ATP exchange did not show the exchange activity between pairs of the other three homologous di- and triphosphonucleosides. On the other hand purified preparations of "amino acid incorporation enzyme" which were able not only to catalyse ADP–ATP exchange, but also to stimulate the incorporation of ^{14}C amino acids into *Alcaligenes* particles, were able to exchange orthophosphate between each pair of homologous nucleotides (Table I).

The exchange reactions are absolutely dependent on the presence of Mg^{++} ions. Mn^{++} only partially replaces Mg^{++} while Ca^{++} has no effect. In the pH range from 5 to 8 the rate of the exchange is essentially constant.

The rate of the exchange reaction is not influenced by a mixture of L-amino acids, by 2,4-dinitrophenol (up to $2 \cdot 10^{-4}$ M) chloramphenicol (up to 0.1 mg/ml) or streptomycin and penicillin at various concentrations. Orthophosphate, purines or pyrimidines added in excess to the incubation mixture had no effect. The failure of monophosphonucleosides at a 20 fold excess each to influence the exchange of diphosphonucleosides with homologous triphosphonucleosides suggests the absence of monophosphonucleoside kinase[7, 8] in the purified "amino acid incorporation enzyme".

Diphosphonucleoside kinase, the enzyme described by BERG AND JOKLIK[6], is able to transfer the terminal phosphate from ATP to heterologous diphosphonucleosides (for example: ATP + UDP ⇌ UTP + ADP) but acts equally well in the presence of Mg^{++}, Mn^{++} or Ca^{++} ions. Since the "amino acid incorporation enzyme" is completely active only in the presence of Mg^{++}, it would appear probable that the two enzymes are different. This was found to be the case. Table II shows that incubation of the purified "amino acid incorporation enzyme" with [^{14}C]ADP, Mg^{++} ions and either CTP, GTP or UTP does not result in appearance of labeled ATP, thus indicating the absence of diphosphonucleoside kinase in the purified enzyme preparation.

In view of the fact that the concentration of the two homologous nucleotides is constant during the exchange reaction, the orthophosphate is probably exchanged according to the following scheme:

$$X - R\text{-}P\text{-}P\text{-}P + E \rightleftharpoons E - P + X - R\text{-}P\text{-}P$$
$$X - R\text{-}P\text{-}P + E - P \rightleftharpoons E + X - R\text{-}P\text{-}P\text{-}P$$

X = purine or pyrimidine

The inability of the "amino acid incorporation enzyme" to exchange the heterologous nucleotides suggests that the enzyme preparation contains four different and specific enzymes, each capable of exchanging the orthophosphate of one pair of homologous di- and tri-phosphonucleosides. In order to differentiate these four reactions a highly purified enzyme preparation was heated at 55° for various periods of time (100 μg of enzyme protein in 1 ml of Tris buffer, 0.05 M, pH 7.3). The exchange for each pair of nucleotides was then determined. The results are presented in the figure (data from Table I).

The thermal inactivation of the "amino acid incorporation enzyme" follows a simple monomolecular law. The rate of inactivation is very different for each exchange reaction, suggesting that four different enzymes are present.

We propose to call these enzymes: adenosine triphosphate-ADP kinase, guanosine triphosphate-GDP kinase, uridine triphosphate-UDP kinase and cytidine triphosphate-CDP kinase.

The formation of a true peptide bond has been demonstrated by SNOKE AND BLOCH[10] with a purified preparation of glutathione synthetase. This enzyme, which catalyses glutathione formation from γ-glutamyl-cysteine and glycine in the presence of ATP and Mg^{++} is also able to promote a rapid exchange of ADP with ATP. These properties of glutathione synthetase suggest that the promotion of amino acid incorporation by the *Alcaligenes* preparation may be due to a similar type of reaction.

It was of interest to determine if glutathione synthetase contained the activity for exchange of other pairs of homologous nucleotides. A preparation of glutathione

TABLE III

EXCHANGE OF LABELED DI- WITH UNLABELED TRIPHOSPHONUCLEOSIDES BY GLUTATHIONE SYNTHETASE

Conditions of incubation as in Table I with 100 μg of glutathione synthetase. Incubation 20 min in air at 34°.

Nucleotides	*Exchange per cent of final equilibrium*	*Rate of exchange μM/mg of protein/h*
[^{14}C]ADP–ATP	90	33
[^{32}P]GDP–GTP	0	0
[^{32}P]UDP–UTP	0	0
[^{32}P]CDP–CTP	0	0
[^{14}C]ADP–ATP (heated enzyme)	1	0

synthetase, kindly supplied by Dr. K. BLOCH, was tested for exchange of all four nucleotides and for amino acid incorporation activity in *Alcaligenes* particles. No effect on incorporation was observed. As shown in Table III, glutathione synthetase did not exchange other diphosphonucleosides with triphosphonucleosides.

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ACETYLCHOLINESTERASE IN FLAGELLATED SYSTEMS

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SUMMARY

1. Cholinesterase could not be detected in *Polytoma uvella*, *Polytomella caeca*, or *Tetrahymena pyriformis* W homogenates.

2. Trout sperm and perch sperm possessed activity corresponding to the splitting of 0.02 to 0.14 μg/h/mg at 37°. This activity was specific for acetylcholine.

3. The enzyme of perch sperm was found to be located in the head of the spermatozoa.

INTRODUCTION

The release and destruction of acetylcholine is an integral part of the nervous control of muscle action, and it is possible that ciliary and flagellar movements also are controlled by a mechanism which utilizes acetylcholine. Thus BÜLBRING *et al.*[1] found that acetylcholine influenced the rate of ciliary movement in the gill plates of *Mytilus edulis* and reported that a homogenate of the tissue contained the enzyme, acetylcholinesterase. SEAMAN AND HOULIHAN[2] examined the protozoon *Tetrahymena gelii* (now *pyriformis*) S and concluded that a specific acetylcholinesterase was present and that the motility of the organism was reduced by eserine and diisopropylfluorophosphate. Pellicular fragments, isolated from a cell homogenate, were later stated to possess all the activity of the cell, and the enzyme was therefore considered[3] to be located in the pellicle. Human semen has been found[4] to have a small cholinesterase activity, but this was said to arise from the seminal plasma. On the other hand SEKINE[5] found that acetylcholine was easily split by both the plasma and the cells of pig semen. MANN[6] mentions work by himself and LEGGE, no details being given, which identifies a specific acetylcholinesterase as being located in the tails of ram sperm. This last result is not in accord with the views of SEAMAN[7] that the enzyme in ciliated systems is associated with a sub-pellicular arrangement of neurofibrils.

In nervous activity the action current is controlled from the site at which acetylcholine is liberated and hydrolysed, and, if a corresponding method of control is used for flagella and cilia the localisation of the enzyme acetylcholinesterase is of first importance. This paper is an attempt to obtain further information on the presence and site of activity in several protozoa.

EXPERIMENTAL

Polytoma uvella *and* Polytomella caeca *homogenates*

Polytoma uvella and *Polytomella caeca* were grown and harvested as previously described[8]. The cells were washed by suspending them once in 0.05 *M* phosphate, pH 7.0, and recovered by centrifuging at 500 × *g* for 5 min. They were then resuspended in 0.05 *M* phosphate, pH 7.0 containing 0.1 *M* sodium chloride and 0.02 *M* magnesium chloride, cooled in an ice–water mixture before and during treatment in an M.S.E. homogeniser (high-speed blade type). Even after 10 min, only a small fraction of the *Polytoma* cells was ruptured, but the majority of the *Polytomella* cells were broken after 2 min treatment. Homogenates were prepared as concentrated as possible and contained up to about 50 mg dry cellular material/ml.

Tetrahymena pyriformis *W homogenates*

These protozoa were grown in a mixture containing 0.2 % "Difco" yeast-extract and 0.2 % "Difco" bacto-tryptone for about five days at 21° and harvested by gentle centrifugation in a Sharples continuous-type centrifuge run at low speed. After washing once with distilled water, they were washed a second time with 0.05 *M* phosphate, pH 7.0, the cells being recovered between washings by spinning at 120 × *g* for 90 sec. The cells, which at this stage were still alive and motile, were suspended in 0.05 *M* phosphate, pH 7.0, containing 0.1 *M* sodium chloride and 0.02 *M* magnesium chloride, cooled, and treated with constant cooling in either an M.S.E. homogeniser at full speed for 2 min or a Potter-Elvehjem type homogeniser for 2 min. The latter was operated by hand in an up-and-down rather than a rotatory manner, and a pinch of powdered quartz was included in the cell suspension. Under these conditions the vast majority of cells were broken up, the homogenate containing 30 to 60 mg dry weight of cellular material/ml.

Brown trout (Salmo trutta) *spermatozoa*

Trout milt was obtained from the Welham Park Fish Hatchery at Malton, Yorkshire, during the December–January period of 1958–59, and was processed at Leeds within a few hours of being expressed from the fish. On dilution with water the sperm invariably became motile, though to varying extents.

Numerous observations in this Department have indicated that, when sperm from the perch (*Perca fluviatilis*) have been allowed to swim, their tails tend to disintegrate, while those of sperm which have not swum remain relatively intact. Since any extraction of enzyme activity during washing had to be reduced to a minimum, it was thought desirable to dilute the milt with a medium in which the cells would not swim. With perch sperm a convenient diluent was 0.05 *M* maleate buffer of pH 6.5[9]. With trout sperm, however, motility persisted when the semen was diluted with such a buffer even down to a pH of 5.5. Sucrose (0.5 *M*) in pH 7.4, 0.05 *M* tris-hydroxymethyl aminomethane was therefore used instead, at a dilution ratio of about 25 : 1. The sperm did not swim on diluting the semen with this solution.

The cells were now spun down (5 min at 1000 × *g*) and washed twice with pH 7.0, 0.05 *M* sodium phosphate, being recovered by centrifugation after each washing. A suspension, containing about 50 mg/ml dry weight of cells, was then made in pH 7.0, 0.05 *M* sodium phosphate containing 0.1 *M* sodium chloride and 0.02 *M* magnesium chloride, and it was this preparation that was used in the measurement of acetylcholinesterase activity. Homogenisation prior to enzyme measurements was omitted, since this process applied to sperm has little effect other than detaching the tails from the heads.

Perch (Perca fluviatilis) *spermatozoan homogenates and fractions*

Live male perch were obtained from the Freshwater Biological Station near Ambleside, Westmorland, during the spawning season from the middle of April to the middle of June, 1959. The fish were kept in tanks filled with running water, and sperm was obtained from them as required.

To prevent the spermatozoa from swimming on dilution, they were diluted with pH 6.1, 0.05 *M* maleate buffer. The use of a buffer of pH 6.5 has been previously reported by the author[9]. Since a few sperm preparations were still fairly motile at this pH, the lower one of 6.1 has been preferred in more recent work. The cells were then centrifuged down (5 min at 1000 × *g*) and washed once with pH 7.0, 0.05 *M* sodium phosphate before being suspended in more pH 7.0, 0.05 *M* sodium phosphate. Although phosphate buffer containing magnesium chloride has been used by SEAMAN AND HOULIHAN[2] and by the author in the first part of the work described here, this system is not very satisfactory, because it causes precipitation of magnesium phosphate. Thus in the later stages at pH 7.4, 0.05 *M* tris-hydroxymethyl aminomethane replaced the 0.05 *M* phosphate.

Since the object of the experiments with perch sperm was to locate the enzyme in either the head or tail, the cells in buffer (without the addition of magnesium and sodium chloride) were treated, with cooling, in an M.S.E. homogeniser for 60 sec. Cell heads, comparatively free from tails, were now obtained by three spinnings at 2000 × *g* for 7 min. Tails were isolated from the supernatant by spinning once at 15000 × *g* for 7 min. This tail fraction was almost entirely free from heads. The original complete homogenate, the cell heads, the tails, and the final supernatant were then made up to equivalent volumes in 0.05 M phosphate or Tris buffer containing 0.1 M sodium chloride and 0.02 M magnesium chloride. Complete homogenates normally contained about 30–40 mg dried cellular material/ml.

Measurement of acetylcholinesterase activity

The suspension (0.9 ml) was incubated at 37° with 0.1 ml of 25 mM acetylcholine, two drops of toluene being added to reduce bacterial growth. Incubation times were usually about 72 h. Acetylcholine remaining in the tubes at the end of this time was estimated by the HESTRIN[10] method, the whole of the incubate being used to give a final volume of 5 ml, which was filtered before measuring the absorption at 540 mμ. Controls were made by incubating acetylcholine with buffer instead of homogenate, and homogenate with buffer instead of acetylcholine.

Possible bacterial breakdown of substrate was also examined. Tubes containing homogenates were held in boiling water for 2 min to inactivate any enzyme activity. After cooling, these tubes were inoculated with bacteria from an agar plate which had been exposed to the atmosphere and then incubated at 37°. In these tubes no breakdown of acetylcholine occurred when toluene was present during incubation with substrate, but some erratic hydrolysis took place when toluene was absent. Furthermore, this erratic hydrolysis appeared to be prevented by 10^{-5} M eserine, indicating a cholinesterase rather than a simple esterase[11].

RESULTS

Polytoma uvella *and* Polytomella caeca

Homogenates of these cells contained no detectable acetylcholinesterase activity. Enzyme activities corresponding to substrate splitting of 0.04 μg/h/mg or more would have been detected in these experiments.

Tetrahymena pyriformis *W*

The S strain of this organism has been examined by SEAMAN AND HOULIHAN[2], and, before undertaking further work, it was thought desirable to make a rapid confirmation of the results of these workers. By using longer incubation times than were used with *Polytoma* and with greater experience of the method, the sensitivity was increased the result being that an activity as low as 0.01 μg/h/mg could be detected.

No splitting could be detected either at 37° or 22° by *Tetrahymena* homogenates.

It was hardly likely that the lack of substrate hydrolysis was due to choline acetylase, since this enzyme requires the presence of acetylcoenzyme A, the supply of which was not likely to be satisfactory in a disrupted cell preparation. This surmise was easily verified by incubating in the presence of 10^{-3} M iodoacetate, at which concentrations choline acetylase is strongly inhibited[12], while acetylcholinesterase, as tested on a gudgeon-muscle brei, was not affected. As expected, no acetylcholine splitting was detected

A natural inhibitor in the cell preparation could conceal the presence of an enzyme. That this did not seem to be so in this particular instance was shown by diluting a perch muscle brei to a concentration which required a day or so at 37° to produce reasonable hydrolysis of acetylcholine. No inhibition of this activity could be produced by incubating this suspension and its substrate with *Tetrahymena* homogenates.

Incubation overnight at 37° with toluene did not, in a gudgeon-muscle brei, result in any inactivation of the acetylcholinesterase, and it thus seemed unlikely that the toluene preservative was affecting the results from *Tetrahymena*.

Control tubes in which *Tetrahymena* homogenates were incubated without the addition of substrate contained material which, on normal development, gave considerable absorption at 540 mμ. Since gudgeon-muscle did not reduce this colour, the absorption could not be due to free acetylcholine. Neither was it due to a bound form of acetylcholine, since neutralised acid extracts of the *Tetrahymena* homogenates contained the colour-producing material, and its intensity was not reduced by incubating the extracts with gudgeon-muscle brei.

Brown trout (Salmo trutta) *spermatozoa*

The results obtained with trout sperm are shown in Table I. With material prepared as described earlier, activities were usually about 0.03 μg/h/mg at 37°. This splitting was considerably reduced by incubation in 10^{-5} *M* eserine, indicating either a specific or a pseudo cholinesterase, but not a common esterase[11]. Butyryl choline was split at a rate lower than the acetyl compound, which indicates a specific

TABLE I

HYDROLYSIS OF CHOLINE ESTERS BY FISH SPERMATOZOA AT 37°

Specimen No. "T" signifies trout spermatozoa· "P" signifies perch spermatozoa	*Acetylcholine chloride split μg/h/mg*	*Acetylcholine chloride split in 10^{-5} M eserine μg/h/mg*	*Butyrylcholine chloride split μg/h/mg*	*Acetyl-β-methyl-choline chloride split μg/h/mg*	*Benzoylcholine chloride split μg/h/mg*
1T	0.04				
5T	0.03				
6T	0.00				
8T	0.04		0.02		
9T	0.04		0.02		
10T	0.02		0.02		
11T	0.03	0.01	0.00	0.01	
12T	0.03	0.01		0.02	0.00
13T	0.03	0.01	0.01	0.01	0.00
14T	0.02	0.01	0.01	0.01	0.01
15T	0.02				
16T	0.00				
16T*	0.06				
17T	0.00				
17T*	0.10				
1P	0.10				
2P	0.13				
3P	0.08				
4P	0.05				
5P	0.15		0.01	0.05	0.02
6P	0.13		0.03	0.02	0.03
7P	0.08	0.00			
8P	0.11	0.00	0.01		
11P	0.06	0.01	0.03	0.05	0.07
12P	0.09	0.00	0.00	0.06	0.03
13P	0.09	0.00	0.00	0.01	0.03

* These preparations were from unwashed sperm, the activity of the plasma being allowed for.

acetylcholinesterase[13]. This was confirmed by the fact that, whereas the enzyme caused little detectable hydrolysis of benzoylcholine, some small breakdown of acetyl-β-methylcholine could be detected when this was used as substrate[14].

The observed substrate hydrolysis was not due to contamination of the cells by traces of seminal plasma, since it was found that when the cells were spun away from the milt the plasma had a lower activity per unit volume than had the milt. Allowing for the activity present in the plasma it was seen (preparations 16T and 17T) that unwashed cells hydrolysed more acetylcholine than did washed cells prepared in the normal way, and it is evident therefore that the enzyme is easily eluted from the cells.

Perch (Perca fluviatilis)

Activities of the homogenates of washed perch sperm are also shown in Table I. Broadly speaking, these results follow the same pattern as those from trout sperm, *i.e.* the hydrolysis of acetylcholine, which was inhibited by eserine, took place at a quicker rate than that of butyrylcholine. The breakdown of acetyl-β-methylcholine and benzoylcholine was also on a reduced scale, but the position regarding the relative magnitudes of the breakdown of these two substrates is confused by the shortage of results and the large errors which occur in the measurement of such small activities.

TABLE II

THE DISTRIBUTION OF ACETYLCHOLINESTERASE IN HOMOGENATES OF PERCH SPERMATOZOA

Figures in columns (a) show acetylcholine chloride split in μg/h/mg at 37°, and in columns (b) acetylcholine chloride split in μg/h/ml at 37°.

Specimen No.	*Total homogenate*		*Cell heads*		*Cell tails*		*Supernatant*	
	(a)	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*
1P	0.10	2.0	0.10	1.8	0.04	0.2	0.15	0.3
2P	0.13	3.9	0.07	1.3	0.19	0.6		
3P	0.08	1.7	0.08	1.3	0.00	0.0		
4P	0.05	2.0	0.07	2.0	0.02	0.2	0.00	0.0
6P	0.13	3.0	0.12	2.5	0.05	0.4	0.11	0.2
7P	0.08	2.9	0.10	2.3	0.04	0.2	0.16	0.5
8P	0.11	3.6	0.10	3.0	0.00	0.0	0.41	1.8
13P	0.09	2.2	0.14	2.5	0.03	0.2		

Table II shows the distribution of enzyme activity between the heads, tails, and supernatant. In each case a given quantity of homogenate was separated into its constituent fractions and these fractions made up to equal volumes. An equivalent quantity of complete homogenate was also made up to the same volume and tested. Columns (a) in Table II show activities per unit weight whilst columns (b) show activities per unit volume. These last figures show directly the contribution which each homogenate fraction makes to the total activity. Most of the enzyme was found in the heads and occasionally a little was extracted into the final supernatant. The contribution arising from the tails was small and in all but one case (preparation 2P) was of about the same magnitude as the experimental error. In any event, small contamination by heads and supernatant could cause the fraction to be slightly active.

DISCUSSION

The inability to detect acetylcholinesterase in *Polytoma* and *Polytomella* may merely reflect the fact that the flagella, and presumably the control mechanism as well, represent only a small part of these cells. Moreover, in these particular experiments the sensitivity of the method was not at its highest. The same may be true of the experiments done with *Tetrahymena*, although in these an activity as low as 0.01 μg/h/mg would have been detected. It is puzzling that SEAMAN AND HOULIHAN[2] were able to detect activity in this organism, albeit a different strain, to the extent of about 0.08 μg/h/mg at room temperature, for such an activity would have easily been detected in our experiments. The smaller incubation times which these authors used must have given Beckmann reading differences which were approaching normal experimental variation. Neither do these authors mention the use of preservative, which was certainly necessary for the long incubations reported here. On the other hand the preservative may have reduced the activity of our preparations, for, although toluene did not appear to cause any inactivation of acetylcholinesterase in gudgeon-muscle brei, it is possible that this result cannot be extrapolated to an enzyme from a different source. The situation here is not clear, but the fact that we have (in unreported experiments) been able to confirm the result of SEAMAN AND HOULIHAN[2] that eserine depresses motility in *Tetrahymena* is in agreement with the presence of an acetylcholine-controlled system.

A cholinesterase, apparently specific for the acetyl compound, was found in trout and perch spermatozoa, where it had an activity corresponding to 0.02 to 0.14 μg substrate hydrolysed/h/mg at 37°. An analysis of the results obtained with perch sperm showed that this activity was located in the heads and not the tails, as might have been expected from the report of MANN[6]. In a private communication MANN has suggested that such differences between results from mammalian sperm and fish sperm do not necessarily mean a disagreement, since these two types exhibit considerable differences in other respects. Whatever the explanation, fish sperm are structurally far simpler than mammalian sperm, and for this reason a consideration of their properties may prove easier to understand.

An acetylcholine-acetylcholinesterase system would be disrupted by glycerol extraction, and therefore it is pertinent to consider the behaviour of extracted cell models. BISHOP[15] has found that glycerol extraction does not destroy rhythmic wave formation and decay in tails of mammalian sperm, but does destroy wave propagation along the flagellum and rotation about the longitudinal axis. This suggests that the acetylcholine-acetylcholinesterase system is more likely to be associated with the control of wave propagation and rotation than with any contraction–relaxation cycles which may exist. In the instances examined by BISHOP[15] these cycles, once initiated, seem to be, if a source of energy (adenosine triphosphate) is present, self-perpetuating.

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STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS

VIII. THE PARTICIPATION OF PROTEIN-BOUND BIOTIN IN THE BIOSYNTHESIS OF FATTY ACIDS

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SUMMARY

One of two purified enzymic components, which catalyze the synthesis of palmitate from acetyl CoA, is rich in protein-bound biotin. Avidin, the egg white protein that specifically binds biotin, severely inhibits fatty acid synthesis. Added free biotin prevents the occurrence of this avidin inhibition. These observations indicate that a biotin-containing enzyme participates directly in fatty acid biosynthesis.

INTRODUCTION

We have previously described[1,2] the preparation and properties of two purified enzyme fractions from avian liver that catalyze the synthesis of palmitic acid from acetyl CoA. The cofactors ATP, TPNH, Mn^{++} and HCO_3^- are absolute requirements for fatty acid biosynthesis in this system. Since the necessity for this spectrum of cofactors could not be explained in terms of a simple reversal of β-oxidation of fatty acids (such as by the system of SEUBERT *et al.*[3]), we proposed[1,2] that a new mechanism for the biosynthesis of fatty acids is operative.

The following abbreviations are used: ATP, adenosine triphosphate; CoA, coenzyme A; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides.

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In the course of examining the two purified enzyme fractions for bound vitamins we discovered that fraction R_{1g} contains an unusually high concentration of protein-bound biotin[4,*]. The present communication provides evidence that the biotin moiety of R_{1g} plays an essential role in fatty acid synthesis.

METHODS AND MATERIALS

Enzymes

Fractions R_{1g} and R_{2g} were obtained in purified form from the clarified supernatant of avian liver homogenates according to previously described methods[1,7]. Acetic thiokinase was prepared from beef heart mitochondria[8], and isocitric dehydrogenase from whole pig heart[9].

Bioassay for biotin

Biotin was assayed microbiologically by the procedure of WRIGHT AND SKEGGS[10]. The growth response of *Lactobacillus arabinosus* measures exceedingly dilute concentrations of *d*-biotin. Digests or samples containing free biotin were added to biotin-deficient media (Difco No. B 419) inoculated with *L. arabinosus* and the mixture was incubated for 24 h at 35°. On the average, in a medium containing 1.0 $\mu\mu$mole of *d*-biotin in 5.0 ml, the O.D. of the bacterial suspension was 0.5 (1.0 cm cell at 660 mμ). Since protein-bound biotin does not assay in this system, release of the biotin moiety by digestion of the protein in 6 N H_2SO_4 for 1 h at 121° (about 15 pounds steam pressure) is necessary.

Avidin

Avidin was prepared from fresh egg white by the method of WOOLEY AND LONGSWORTH[11] with the following modifications: operational temperatures were held below 5°, separation of fractions, was made by centrifugation, and distilled water was used throughout. Avidin was assayed by direct addition of aliquots (diluted in sterile 0.9 % saline) to the microbiological assay system used for biotin determination. In general, the limiting biotin binding capacity of the purified avidin preparations (*i.e.* in the presence of excess biotin) was 6 $\mu\mu$moles of biotin/μg avidin protein. Avidin prepared by this method already contained bound biotin to the extent of 1 $\mu\mu$mole/μg protein or one sixth of its total binding capacity.

Assay for fatty acid biosynthesis

As in previous studies[1,7] the measure of fatty acid synthesis was either the rate of incorporation of [1-^{14}C]acetyl CoA[*] (or acetate) into long-chain fatty acids, or the rate of TPNH oxidation. Protein was estimated by the biuret reaction[12].

Chemical reagents

Acetyl CoA and TPNH were prepared as previously described[1,7]. Other nucleotide

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[***] Preliminary results of this investigation were presented before the 133rd meeting of the American Chemical Society, San Francisco, April, 1958[5] and before the American Society of Biological Chemists, Philadelphia, April, 1958[6].

[*] An alternate system composed of acetate, CoA, cysteine, ATP, isocitrate, TPN^+, Mn^{++} and HCO_3^- may be used provided that R_{1g} and R_{2g} are supplemented with purified acetic thiokinase and isocitric dehydrogenase.

reagents and *d*-biotin were obtained from commercial sources. Biocytin (ε-N-biotinyl-L-lysine)[13] was the gift of Dr. K. FOLKERS of the Research Laboratories of Merck Sharp and Dohme. The trypsin employed for proteolytic digestion of R_{1g} was brand "1–300" of Nutritional Biochemical Company.

RESULTS

Biotin content of R_{1g}

Fractions R_{1g} and R_{2g} are the only enzyme components needed for fatty acid synthesis from acetyl CoA. These two purified fractions were analyzed by microbiological assay for each of several protein-bound vitamins. A remarkably high concentration of biotin was discovered in fraction R_{1g}. Ten different preparations of R_{1g} obtained from chicken liver contained protein-bound biotin in the concentration range of 150 to 250 mμg/mg of protein. These values correspond approximately to one mole of biotin/10^6 g of protein. R_{2g}, by comparison, possessed very little biotin (< 2.0 mμg/mg).

All the protein fractions obtained during the purification of R_{1g}, beginning

TABLE I

INCREASE IN PROTEIN-BOUND BIOTIN CONCENTRATION DURING PREPARATION OF R_{1g}

Fraction	Preparation I		Preparation II	
	mμg biotin / mg protein	*% recovery of biotin*	*mμg biotin / mg protein*	*% recovery of biotin*
Supernatant	8	100	5	100
0–30% Ammonium sulfate	19	32	28	61
Gel eluate	64	22	115	9
R_{1g}	215	18	215	9

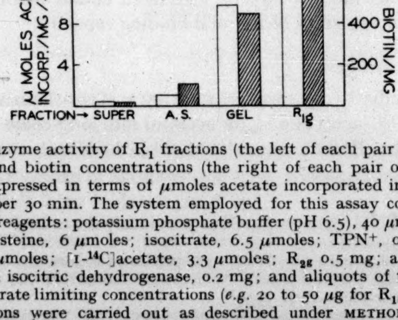

Fig. 1. The specific enzyme activity of R_1 fractions (the left of each pair of columns) is compared with the protein-bound biotin concentrations (the right of each pair of columns). The specific enzyme activity is expressed in terms of μmoles acetate incorporated into fatty acid per mg of R_1 fraction protein per 30 min. The system employed for this assay contained in a volume of 0.50 ml the following reagents: potassium phosphate buffer (pH 6.5), 40 μmoles; $MnCl_2$, 0.3 μmole; ATP, 2.7 μmoles; cysteine, 6 μmoles; isocitrate, 6.5 μmoles; TPN^+, 0.4 μmole; CoASH, 0.05 μmole; $KHCO_3$, 10 μmoles; [1-^{14}C]acetate, 3.3 μmoles; R_{2g} 0.5 mg; a preparation containing acetic thiokinase and isocitric dehydrogenase, 0.2 mg; and aliquots of the various R_1 fractions from chicken liver at rate limiting concentrations (*e.g.* 20 to 50 μg for R_{1g}). Biotin analyses of the same set of R_1 fractions were carried out as described under METHODS. Fractions: Super = 100,000 × *g* supernatant; A.S. = 0–30% ammonium sulfate; Gel = gel eluate; R_{1g} = reprecipitated eluate.

with the centrifugal supernatant of chicken liver homogenate[1], were analyzed for biotin. The biotin content only of the R_1 fractions progressively increased with purification (*cf.* Table I). About 10–20 % of the biotin in the crude supernatant was recovered in R_{1g}. Further studies revealed that the specific enzyme activity of an R_1 fraction (assayed with an excess of R_{2g} in the complete fatty acid synthesizing system) is directly proportional to the biotin content of the fraction (*cf.* Fig. 1). The close correlation of biotin content and activity clearly suggests that the rate of fatty acid synthesis, under these conditions, is limited only by a biotin-containing component in fraction R_{1g}.

Nature of the biotin moiety

Acid hydrolysis (6 *N* sulfuric acid at 121° for 1 h)[10] was the only successful method found for releasing free biotin from R_{1g}. Neither untreated R_{1g} nor trypsin digests of the enzyme fraction (see below) supported growth of *L. arabinosus*. In order to prove that the active principle released by acid hydrolysis of R_{1g} was biotin, the digests of two preparations of R_{1g} were chromatographed on paper in three solvent systems[14] (Table II). The chromatogram of one sample of digest resolved with 70 % ethanol is presented in Fig. 2. The R_F of the biotin isolated from R_{1g} was identical with that of *d*-biotin*.

TABLE II

CHROMATOGRAPHY OF ACID DIGEST OF R_{1g}

Aliquots of *d*-biotin and neutralized sulfuric acid digests of R_{1g} (each containing approximately 5 μμmoles of biotin) were chromatographed separately and in mixture on Whatman No. 1 filter paper sheets in three different solvent systems. Segments were cut from the developed sheets and assayed for biotin directly.

R_{1g} *Preparation*	*Solvent*	R_F *Biotin*	R_F *Digest*	R_F *Mixture*
A	70 % Ethanol	0.84–0.87	0.84–0.87	0.84–0.87
	n-Butanol (sat. with water)	0.42–0.50	0.39–0.50	0.42–0.50
D	70 % Ethanol	0.77–0.81	0.77–0.81	0.77–0.81
	95 % Isobutyric acid	0.83–0.89	0.79–0.86	0.83–0.89

Digestion of R_{1g} with crude trypsin leads to the release not of free biotin, but rather of soluble bound forms of biotin that cannot support the growth of *L. arabinosus*. Free biotin may subsequently be split from the soluble fragments by acid hydrolysis (*cf.* Table III). Combined tryptic and acid digestion is more effective than acid digestion alone for the release of free biotin. The results of paper chromatographic studies indicate that many different soluble bound-biotin components[17], including possibly, biocytin[13] (by comparison of R_F values), are released during tryptic digestion.

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* Oleic acid will substitute for biotin in the growth of *L. arabinosus*[15, 16], but at concentrations 10^5 greater than biotin under the conditions of this bioassay. Although it is possible that R_{1g} contains exceedingly small quantities of oleic acid, there has been no evidence to suggest that oleic acid has interfered with the biotin determinations.

Neither heating samples of R_{1g} up to 5 min in a boiling water bath, nor denaturation of the protein in perchloric acid (0.2 *M*) releases free biotin. These findings emphasize that the biotin moiety is bound by covalent linkage to the protein of R_{1g}.

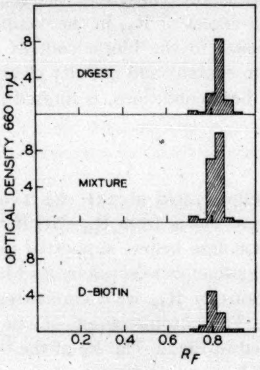

Fig. 2. The chromatographic identification of biotin isolated from R_{1g} with free *d*-biotin. The R_F values were measured in a 70% ethanol system. See Table II. The ordinates represent biotin concentration which is measured here as O.D. at 660 mμ in the *L. arabinosus* bioassay system (see METHODS).

TABLE III

RELEASE OF BIOTIN FROM R_{1g} BY TRYPTIC DIGESTION

Aliquots of R_{1g} each containing 2.5 mg of protein were treated as indicated below. Samples 4, 5 and 6 were incubated with 2.5 mg crude trypsin at room temperature (final volume 0.10 ml) and then were placed in a boiling water bath for 15 sec. After cooling, the protein coagulum was separated by centrifugation and was then digested with H_2SO_4 in the usual fashion and the biotin content determined in the *L. arabinosus* assay. The supernatant was assayed both before and after acid digestion.

	(μμmoles biotin)			
Treatment	*Clarified supernatant after boiling (no digestion with acid)*	*Clarified supernatant after boiling (digested with acid)*	*Precipitate of boiled sample (digested with acid)*	*Total free biotin*
1. No				3
2. Acid digestion only				602
3. Boiled 15 sec	3	128	563	694
4. Trypsin 12′	4	241	540	785
5. Trypsin 40′	8	580	390	978
6. Trypsin 100′	24	788	252	1064

Avidin inhibition of fatty acid syntheiss

Since the close correlation of the bound biotin content with the enzymic activity of R_{1g} indicates that biotin is required for fatty acid synthesis, it follows that synthesis would be impaired if the biotin moiety were blocked. Avidin, a protein that can be highly purified from egg white, has traditionally been noted for its apparently selective affinity for biotin[18]. Consequently avidin was prepared in purified form[11] and added to the fatty acid synthesizing system. The data presented in Table IV show that microgram quantities of purified avidin efficiently inhibit the incorporation of acetate into long-chain fatty acids, (*cf.* also Fig. 4). Likewise the oxidation of TPNH that accompanies synthesis[2] is greatly diminished in the presence of avidin (*cf.* Fig. 3).

TABLE IV

INHIBITION OF ACETATE INCORPORATION BY AVIDIN AND REVERSAL OF INHIBITION BY *d*-BIOTIN

Avidin added (μg)	*Fatty acid synthesis** *(mμmoles acetate incorporated in 30 min)*	
	No biotin added	*12 mμmoles biotin added*
0	87	77
3.2	74	81
9.6	55	75
32	45	79
64	29	71

* Experimental conditions are the same as in Fig. 4.

If the inhibiting effect of avidin were due solely to its biotin binding capability then free biotin should prevent this inhibition. Fig. 4 (left hand side) contains a plot of the extent of fatty acid synthesis as an inverse function of avidin concentration in the system. In the right hand side of Fig. 4 the rate of fatty acid synthesis as a

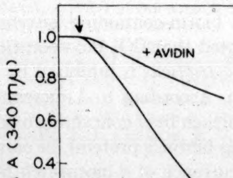

Fig. 3. Avidin inhibition of fatty acid synthesis as measured by TPNH oxidation. Purified avidin (46 μg) was added to one of two identical systems each of which contained in a total volume of 0.50 ml the following reagents: potassium phosphate buffer (pH 6.5), 50 μmoles: $MnCl_2$, 0.3 μmole; ATP, 1.0 μmole; acetyl CoA, 0.21 μmole; $KHCO_3$, 5.0 μmoles; TPNH, 0.17 μmole and R_{2g}, 1.5 mg. The reaction was initiated in both systems by adding 0.26 mg R_{1g} (indicated by arrow). The O.D. at 340 mμ (1.0-cm cell) was followed continuously in the Beckman DUR spectrophotometer (at 38°).

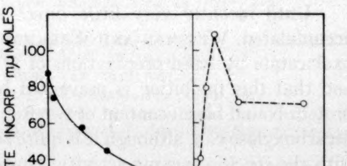

Fig. 4. Prevention of avidin inhibition of fatty acid synthesis by added *d*-biotin. In the left curve increasing amounts of purified avidin are added to a series of identical incubation mixtures. The maximum inhibitory effect in this sequence was obtained with 64 μg avidin. The right curve demonstrates that by adding increasing small quantities of free *d*-biotin to a second series of incubation mixtures each containing 64 μg avidin, the inhibitory effect of avidin on synthesis is progressively eliminated. Each experimental system contained in a volume of 0.50 ml the following reagents: potassium phosphate buffer (pH 6.5), 40 μmoles; $MnCl_2$, 0.3 μmoles; cysteine, 6.0 μmoles; ATP, 2.7 μmoles; TPN, 0.38 μmoles; isocitrate, 6.0 μmoles; CoASH, 0.04 μmoles; [1-^{14}C]acetate, 1.32 μmoles; $KHCO_3$, 10 μmoles; a preparation containing acetic thiokinase and isocitric dehydrogenase, 0.3 mg; and R_{2g}, 0.9 mg. The reactions were initiated by adding 0.24 mg R_{1g}. Incubations were carried out at 38° for 30 min under an atmosphere of nitrogen. Fatty acids were isolated in the usual manner[7].

function of free biotin concentration in the presence of avidin is plotted. Thus *d*-biotin in minute concentrations can prevent the avidin inhibition of acetate incorporation. These observations support the idea that avidin inhibits exclusively by virtue of its biotin-binding capacity*.

DISCUSSION

Although biotin is necessary for the maintenance or growth of probably all living forms, the nature of its biological function has never been satisfactorily elucidated. Biotin has been implicated in various enzyme systems primarily on the basis that the enzyme activity in question is selectively diminished in the biotin deficient organism. A majority of the enzyme systems so implicated appear to be concerned with bicarbonate metabolism[20]. These systems are involved respectively in (a) decarboxylation of oxalacetate and incorporation of CO_2 into oxalacetate[21–24], (b) oxidative decarboxylation of malate to pyruvate (malic enzyme)[23], (c) synthesis of citrulline from ornithine[25,26], (d) carboxylation of propionyl CoA[20,27], β-hydroxyisovaleryl CoA[28,29] and β-methyl crotonyl CoA[30].

Some of the above enzymes have been extensively purified (malic enzyme[23], ornithine transcarbamylase[26] and propionyl CoA carboxylase[37]). Yet no significant quantities of protein-bound biotin could be detected. This paradox has led to the hypothesis that biotin functions in the synthesis of these enzymes and not as a prosthetic group[31].

Until recently very little direct evidence for biotin-containing enzymes has accumulated. WESSMAN AND WERKMAN[21] demonstrated that CO_2 incorporation into oxalacetate by lysed preparations of *Micrococcus lysodeikticus* is inhibited by avidin and that this inhibition is prevented by free biotin. According to LICHSTEIN[24] the protein-bound biotin content of two fractions from chicken liver containing oxalacetic decarboxylase can, although it is quite small (0.002 μg biotin/g protein), be correlated with the specific enzyme activity. However, the occurrence of a biotin-rich fraction (250 μg biotin/g protein), which is essential for fatty acid synthesis and which is selectively inhibited by avidin, supports the contention that biotin functions as a prosthetic group[4]. More recently LYNEN *et al.*[30] found that a biotin-containing enzyme from bacteria catalyzes the carboxylation of β-methyl crotonyl CoA (see [29]). According to their evidence CO_2 probably enters into an enzyme-biotin-CO_2 complex.

Biotin has been previously implicated in the synthesis of oleic acid by WILLIAMS AND FIEGER[15] on the basis that the requirement for biotin by *Lactobacillus casei* is abolished by oleic acid in high concentrations (10^5 × biotin). The explanation for this observation is probably complex, since biotin can be isolated from these microorganisms when these are grown in the absence of biotin but in presence of oleic acid[32]. Also animal nutritional studies[33,34] have suggested that biotin deprivation leads to impaired lipide metabolism. A recent investigation[35] has shown that the total amount of R_{1g} (as assayed in the fatty acid synthesizing system) that can be obtained from the livers of biotin-deficient rats and chicks is less than that in the control animals. Nevertheless, the biotin content and the specific enzyme activity of the purified fraction is nearly the same, whether the source is the normal or the biotin-deficient chicks.

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* The binding of avidin[19] by protein-linked biotin appears to be irreversible in the sense that large concentrations of free biotin do not relieve the inhibition of synthesis if added to the system after avidin and R_{1g} have been incubated together. Biotin, biocytin and biotin in the forms released during tryptic digest of R_{1g} cannot replace R_{1g} in the purified enzyme system.

Bicarbonate is an absolute requirement for fatty acid biosynthesis[1]. This fact had suggested that the biotin moiety of R_{1g} is concerned with the utilization of bicarbonate in the system. Our laboratory[36] has recently demonstrated that R_{1g} catalyzes the synthesis of malonyl CoA in the presence of ATP, HCO_3^- and acetyl CoA. Since the enzymes contained in fraction R_{2g} suffice for the synthesis of palmitate from malonyl CoA (and acetyl CoA), the biotin-containing R_{1g} must be concerned exclusively with carboxylase activity.

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INTERRELATIONSHIPS BETWEEN THE METABOLISM OF SHORT-CHAIN FATTY ACIDS BY RUMINANT LIVER SLICES*

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SUMMARY

The metabolism of acetate, propionate, butyrate and isobutyrate by liver slices from starved sheep were investigated. The ^{14}C from carboxyl-labeled acetate and carboxyl-labeled butyrate was found in carbon dioxide, protein and lipids. No ^{14}C could be recovered in ketone bodies. When labeled propionate was used, ^{14}C was found in carbon dioxide, glucose, protein, succinate, malate and fumarate, but the amount of ^{14}C recovered in lipids was negligible. Propionate inhibited the conversion of acetate to carbon dioxide, but the metabolism of propionate was stimulated by presence of either acetate or glucose. The presence of butyrate caused a marked stimulation in the metabolism of propionate but depressed the metabolism of acetate. Propionate had no effect on butyrate metabolism. Citrate stimulated the metabolism of butyrate but not propionate. Evidence was presented to show that it was unlikely that the stimulatory effect of butyrate on propionate metabolism could be explained on the basis of energy contributions from butyrate.

INTRODUCTION

Although the simultaneous production of several short-chain fatty acids in the rumen is well established[1], interactions between the metabolism of these acids by the body tissues have received little attention.

Several investigators have observed, both in the intact animal[2–4] and in rumen epithelial tissue[5–7], that propionate depressed the ketogenic effect of butyrate and of acetate. ARMSTRONG AND BLAXTER[8] noted that when a mixture of acids was administered to sheep, the presence of a small amount of either propionate or butyrate facilitated the metabolism of acetate and prevented the high heat increment that occurred when only acetate was administered. PENNINGTON AND PFANDER[6] found that the uptake of acetate by rumen epithelial tissue was increased by the presence of butyrate and slightly decreased by the addition of propionate. The uptake of butyrate was increased when propionate was present but was inhibited by acetate.

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These workers also reported that the uptake of propionate was strongly inhibited by the presence of butyrate. All of these findings suggest that, in the ruminant animal, the metabolism of one member of the volatile fatty acid series may be modified by the presence of another acid.

This study was undertaken to study the effects of various metabolites on the metabolism of acetate, propionate and butyrate by ruminant liver.

MATERIALS AND METHODS

The general procedure used in this study consisted of incubating ^{14}C labeled acids in the presence of buffered tissue slices with and without the various cosubstrates. Following the incubation period, the end products were isolated and their ^{14}C content determined.

Labeled substrates

The ^{14}C substrates used were uniformly labeled glucose; carboxyl-labeled sodium acetate, sodium propionate, sodium butyrate and sodium isobutyrate and sodium citrate labeled in the alpha carboxyl groups. Citric acid was purchased from Tracerlab, and the other radio compounds were purchased from the Nuclear Chicago Corporation.

Tissue preparation

Sheep, starved 6 to 8 days, were killed by exsanguination, the livers removed immediately, and slices prepared using a Stadie Riggs microtome[9]. The liver tissue was held in Krebs–Ringer bicarbonate buffer[10] pH 7.0 at 0° at all times, except while in the microtome, until the incubation was commenced.

Incubation

The incubations were carried out in 250-ml Warburg flasks at 37° under an atmosphere of 95 % oxygen, 5 % carbon dioxide. Each flask contained 3 g. wet wt., of liver slices, ^{14}C labeled substrate and sufficient Krebs-Ringer bicarbonate buffer, pH 7.0, to make a final volume of 20 ml. The molar concentrations were adjusted in flasks containing a cosubstrate to keep an equal concentration of acids in all flasks. Glucose or butyrate were added at half the concentration of the other cosubstrates on the basis of C_2 units contributed to the citric acid cycle.

The reactions were stopped after a 3-h incubation period by the addition of perchloric acid from the side arm of the flasks[11].

Separation of metabolic end products

The carbon dioxide produced during the incubation period was trapped in alkali in the center well of the flask. At the end of the incubation period, the contents were washed into a test tube with boiled water. The carbonate was precipitated as the barium salt and filtered onto a paper disc, washed successively with water, acetone and ether and dried under an infrared lamp.

The incubation mixtures which had been deproteinized by perchloric acid, were homogenized with a Servall Omni-mixer for 30 sec, then centrifuged for 10 min at 1500 rev./min. The residue was re-extracted twice with a volume of 0.33 *N* perchloric acid and retained for the separation of lipids and protein. The combined supernatant and washings were neutralized with 2 N KOH, cooled, and the potassium perchlorate which precipitated was removed by filtration.

Glucose was isolated as the phenylosozone from an aliquot of the filtrate[12]. The glucoseosozone crystals were washed 3 times with water, recrystallized from ethanol, filtered onto a filter paper disc, washed with ether, and dried.

The remaining filtrate was concentrated to less than 5 ml on a flash evaporator and the di- and tri-carboxylic acids were separated by chromatography on a Dowex-1 formate column[11]. The tubes containing the effluent in 2-ml fractions were frozen on a slope to allow maximum surface exposure, then dried in a freeze-dry apparatus. The volatile fatty acids were not retained in samples exposed to this procedure, but the di- and tri-carboxylic acids were almost completely recovered. The residue in each tube was dissolved in water, plated onto an aluminum planchet, and dried under an infrared lamp. The acids were identified by the order that the radioactivity peaks were eluted from the Dowex-1 column and the identity of the individual acids was verified by descending paper chromatography[13].

In some of the experiments, the poly-carboxylic acid fraction was not separated into individual acids, but was eluted from the resin in one fraction with 6 N formic acid. This fraction was lyophilized, the residue dissolved in water and an aliquot dried in an aluminum planchet. The residue following the perchloric acid treatment was separated into the lipid and protein fractions using the method of KATZ AND CHAIKOFF[14]. The final hexane phase, containing the total lipids, was plated directly onto aluminum planchets and dried. The defatted residue, considered protein, was pressed into a pellet of 1.8 cm diameter in a hydraulic press under a pressure of 4000 Lbs./in^2.

Counting procedure

The ^{14}C content of the various samples was determined by measuring the time required to reach 2560 counts with a Nuclear-Chicago Corporation D-47 Geiger counter. Corrections for self absorption to infinite thinness were made on the barium carbonate and protein samples, whereas the samples plated directly onto the aluminum planchets were assumed to have infinite thinness. The values were expressed as the total counts per minute (cpm) per flask.

RESULTS

Effects of cosubstrates on the metabolism of acetate

The results of experiments conducted to study the effects of cosubstrates on the incorporation of ^{14}C from [1-^{14}C]acetate into carbon dioxide, glucose, protein and lipids are shown in Table I. The presence of either propionate or butyrate depressed the incorporation of ^{14}C into carbon dioxide from [1-^{14}C]acetate, whereas the addition of glucose caused a slight stimulation. The addition of propionate to [1-^{14}C]acetate had no effect on the ^{14}C incorporated into lipids and protein. The ^{14}C recovered in the glucose fraction was negligible. The production of ketone bodies could not be detected by either a colorimetric method[5,15] or by the incorporation of ^{14}C into ketone bodies.

Effects of cosubstrates on the metabolism of butyrate

The results of three experiments conducted to determine the effects of co-

TABLE I

EFFECT OF COSUBSTRATES ON THE METABOLISM OF [1-^{14}C]ACETATE

Each flask contained 200 μmoles acetate and 5 μC of [1-^{14}C]acetate. The cosubstrates added were: 200 μmoles propionate, 100 μmoles butyrate and 100 μmoles glucose. The control flask contained an additional 200 μmoles acetate.

Fraction	Expt. No.	Cosubstrate			
		None *counts/min/flask*	*Propionate* *counts/min/flask*	*Butyrate* *counts/min/flask*	*Glucose* *counts/min/flask*
CO_2	1	27,818	1,438	—	—
		22,758			
	2	19,865	7,952	—	—
	3	6,260	—	2,655	—
		6,785	—	1,832	—
	4	9,589	—	—	10,654
Glucose	1	18	0	—	—
		46	3	—	—
	2	110	53	—	—
Protein	1	794	750	—	—
		1,108	792	—	—
	2	1,091	1,132	—	—
Lipid	1	766	757	—	—
		701	681	—	—
	2	1,595	1,408	—	—

TABLE II

EFFECT OF COSUBSTRATES ON THE METABOLISM OF [1-^{14}C]BUTYRATE

All flasks contained 100 μmoles butyrate containing 2.5 μC of [1-^{14}C]butyrate. The cosubstrates added were: 200 μmoles propionate, 200 μmoles acetate and 200 μmoles citrate. The control flask contained an additional 100 μmoles butyrate.

Fraction	Expt. No.	Cosubstrate			
		None *counts/min/flask*	*Propionate* *counts/min/flask*	*Acetate* *counts/min/flask*	*Citrate* *counts/min/flask*
CO_2	5	16,456	15,928	—	—
	6	21,244	25,049	22,157	52,860
	7	9,894	11,177	—	—
Glucose	5	93	87	—	—
	6	90	170	48	402
Protein	5	455	779	—	—

substrates on the incorporation of ^{14}C from [1-^{14}C]butyrate into carbon dioxide, glucose and protein are shown in Table II. The incorporation of ^{14}C into carbon dioxide from [1-^{14}C]butyrate was not affected significantly by propionate or acetate but was increased by the presence of citrate. The incorporation of ^{14}C into glucose and protein fractions followed a pattern similar to that for the carbon dioxide. As with carboxyl-labeled acetate, no ketone bodies were detected by either of the two methods.

Effects of cosubstrates on the metabolism of propionate

The results of four experiments which were conducted to determine the effects of cosubstrates on the incorporation of ^{14}C from [1-^{14}C]propionate into CO_2, glucose, the acids of the citric acid cycle, protein and lipids are shown in Table III.

TABLE III

EFFECT OF COSUBSTRATES ON THE METABOLISM OF [1-^{14}C]PROPIONATE

All flasks contained 200 µmoles propionate with 1 µC of [1-^{14}C]propionate. The cosubstrates added were: 100 µmoles butyrate, 200 µmoles acetate, 100 µmoles glucose and 200 µmoles citrate. The control flask contained an additional 200 µmoles propionate.

Fraction	*Expt. No.*	*Cosubstrate*				
		None counts/min/flask	*Butyrate counts/min/flask*	*Acetate counts/min/flask*	*Glucose counts/min/flask*	*Citrate counts/min/flask*
CO_2	8	12,903	—	15,004	—	—
	9	33,904	—	59,704	—	—
	10	4,634	31,643	20,671	17,072	6,106
	11	43,212	140,045	71,168	77,223	53,106
Glucose	9	92	—	181	—	—
	10	462	1,606	2,094	2,232	428
	11	68	344	109	119	227
Malate	10	3,591	22,268	11,190	10,128	4,449
	11	11,974	79,960	30,125	41,628	26,575
Succinate	10	5,052	26,434	12,882	12,903	1,834
	11	12,406	35,952	20,581	29,430	6,200
Fumarate	10	747	5,736	2,457	2,037	827
	11	1,600	24,318	3,950	8,250	3,650
Protein	10	557	833	467	625	617
	11	459	902	537	718	481
Lipid	9	12	—	36	—	—
	10	37	76	71	59	52
	11	4	49	4	21	49

The presence of butyrate caused a marked stimulation on the incorporation of ^{14}C into carbon dioxide from [1-^{14}C]propionate. Both acetate and glucose caused an increased incorporation of ^{14}C into carbon dioxide, but neither of these increases was as great as that obtained with butyrate. The addition of citrate had essentially no effect upon the ^{14}C recovered in the carbon dioxide fraction from [1-^{14}C]propionate.

Acetate, glucose and butyrate all exerted some stimulating effect upon the ^{14}C incorporated into glucose, but citrate had little effect.

Three dicarboxylic acids were isolated, succinic acid, malic acid and fumaric acid. The incorporation of ^{14}C into all three of these acids was greater when butyrate was included as a cosubstrate than with the other cosubstrates used. Acetate and glucose showed some stimulatory effect upon the incorporation of ^{14}C into the three acids, but citrate had a depressing effect on ^{14}C-incorporation into succinic acid.

The addition of either butyrate or glucose as a cosubstrate with [1-^{14}C]propionate had a slight stimulatory effect upon the incorporation of ^{14}C into protein. Neither acetate nor citrate had any appreciable effect. The incorporation of ^{14}C into lipids from [1-^{14}C]propionate was negligible.

Inasmuch as the concentrations of the cosubstrates were equalized on the basis of their contribution of C_2 units to the citric-acid cycle, the effects of different cosubstrates are comparable. The stimulation of propionate metabolism caused by acetate and glucose was consistently less than the stimulation caused by butyrate.

Relative metabolism of cosubstrates

Three experiments were conducted to determine the rates of metabolism of the various cosubstrates in the presence of propionate. In the third experiment the effect of butyrate and propionate on carboxyl-labeled isobutyrate was also studied. The results of these experiments, expressed as percentage of ^{14}C incorporated into carbon dioxide, are shown in Table IV. The extent of formation of labeled carbon dioxide from the carboxyl groups of butyrate, citrate and isobutyrate was essentially the same. However, the percentage of ^{14}C incorporated into carbon dioxide from either carboxyl-labeled acetate or uniformly labeled glucose was considerably less than that from the other acids.

TABLE IV

PERCENTAGE OF ^{14}C INCORPORATED INTO CARBON DIOXIDE

Substrates*		Experiment		
		12 %	13 %	14 %
[1-^{14}C]butyrate	+ propionate	4.41	5.72	3.14
[1,5-^{14}C]citrate	+ propionate	5.32	3.62	—
[1-^{14}C]acetate	+ propionate	0.18	0.41	—
[^{14}C]glucose	+ propionate	—	0.34	—
[1-^{14}C]isobutyrate	+ propionate	—	—	5.91
[1-^{14}C]isobutyrate		—	—	8.46
[1-^{14}C]isobutyrate	+ butyrate	—	—	8.14

* The following amounts of substrates were used per flask: butyrate and glucose 100 μmoles; propionate, citrate, acetate and isobutyrate 200 μmoles. The ^{14}C levels per flask: 2.5 μC for butyrate, citrate and isobutyrate; 5 μC for glucose and acetate.

The appearance of labeled carbon into carbon dioxide from ^{14}C isobutyrate was not altered by the presence of butyrate as a cosubstrate. When propionate was the cosubstrate, however, the amount of ^{14}C found in the carbon dioxide was decreased.

DISCUSSION

In the investigations reported, carboxyl-labeled acetate, propionate, butyrate and isobutyrate were all rapidly metabolized by slices from ruminant liver. The ^{14}C from the labeled acetate was found largely in the carbon dioxide, lesser amounts in the lipids and protein, and negligible amounts in carbohydrate. The distribution of ^{14}C from carboxyl-labeled butyrate followed a pattern similar to that of acetate. When labeled propionate was incubated with liver slices, ^{14}C was readily incorporated into carbon dioxide, acids of the citric acid cycle, glucose and protein, but the amount of ^{14}C recovered in the lipids was negligible. All of these findings are in accord with expectations based upon known pathways of intermediary metabolism.

In contrast with expectations, however, no detectable synthesis of ketone bodies occurred during the incubation of either acetate or butyrate with the liver slices. These results are discordant with the findings of ketone body production from acetate and butyrate in the whole animal[2–4]. PENNINGTON[16] found ketone body production from acetate and butyrate by sheep-liver slices, but the amount produced was small compared to that produced by rat-liver slices. On the other hand, MCCARTHY *et al.*[17] found that ketone bodies were not produced from labeled acetate or butyrate in perfused ruminant liver. Although the presence of propionate in the perfusing medium might account for the lack of ketone body production in the latter work[16], such an explanation would not hold for the work reported herein, as no ketone bodies were found even when acetate or butyrate were the sole substrates.

The inhibition of the production of labeled carbon dioxide from carboxyl-labeled acetate by propionate and butyrate (Table I) is consistent with the observations of others[16, 18–20]. Since propionate does not inhibit the production of labeled carbon dioxide from [1-^{14}C]butyrate (Table II)[16, 19], it would seem that the site of inhibition must occur early in the metabolic pathway of acetate. PENNINGTON AND APPLETON[20] indicated that propionate inhibits the formation of acetyl-CoA from acetate. Such a mechanism, however, would not explain the lack of propionate inhibition on the formation of labeled lipids and protein from acetate (Table I). Moreover, if propionate and acetate are activated by the same enzyme in sheep liver as is the case for ox heart[21], pig heart[22], and yeast[23], one would expect acetate to inhibit the formation of propionyl-CoA from propionate. Instead of inhibiting propionyl-CoA formation, the presence of acetate stimulated the metabolism of propionate (Table III).

The marked stimulation of propionate metabolism by butyrate and to a lesser degree by acetate and glucose (Table III) is surprising. These findings are contrary to the observations with rat liver[19] where neither acetate nor butyrate had an effect on the conversion of propionate to CO_2 and those with rumen epithelium[6] where no effect was observed by either acetate or glucose on propionate uptake and where butyrate depressed propionate uptake. FRIEDBERG, ADLER AND LARDY[24] found that the addition of β-hydroxybutyrate to rat-liver mitochondria resulted in a marked stimulation of the carboxylation of propionyl-CoA. They further showed that the β-hydroxybutyrate merely served as an easily oxidizable substrate providing the ATP necessary for the carboxylation reaction. A similar explanation for the results reported herein is unlikely because liver slices rather than mitochondria were used and because no stimulatory effect of citrate on propionate metabolism was observed (Table III) even though citrate was oxidized as well as butyrate (Table IV).

It is clear from the results presented herein as well as those of others[16, 18–20] that multiple interactions occur in the metabolism of volatile fatty acids. The mechanisms whereby these interactions operate and their role in the regulation of the metabolism of the short chain fatty acids should be fruitful areas of future investigations.

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STIMULATION OF PROPIONATE METABOLISM BY MONOCARBOXYLIC ACIDS*

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SUMMARY

Butyrate stimulates the metabolism of propionate in liver from rats and non-fasted sheep, though to a lesser extent than fasted sheep. The butyrate stimulatory effect is exerted on the carboxylation of propionate but not propionyl-CoA. Isobutyrate, β-hydroxybutyrate, valerate, hexanoate and octanoate all stimulate propionate metabolism, but citrate, succinate and α-ketoglutarate are ineffective. It is postulated that the stimulative effect operates via a CoA transphorase.

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INTRODUCTION

In the preceding paper[1] it was shown that butyrate exerts a marked stimulative effect on the metabolism of propionate by liver slices from fasted sheep. A stimulatory effect is surprising since the usual effect of one acid on another is that of inhibition[2–5]. The results of further investigations into the nature of the stimulative effect are presented in this paper.

MATERIALS AND METHODS

Tissue preparations and incubation procedure

Liver from adult sheep and adult rats was used in this study. Tissue slices were prepared and handled as previously described[1]. Acetone powders of sheep liver were prepared according to the method of MORTON[6].

Incubations of liver slices were carried out in 250-ml Warburg flasks as previously described[1]. The amounts of the various substrates used were equalized on the basis of acetate units contributed to the citric acid cycle. The labeled substrates used were [1-^{14}C]propionate and [^{14}C]bicarbonate.

Propionyl-CoA and butyryl-CoA were prepared from their respective anhydrides and coenzyme A according to the method of SIMON AND SHEMIN[7]. These preparations were stored at —15° until used.

Isolation and counting procedures

In the experiments using labeled bicarbonate, the residual bicarbonate was removed at the end of the incubation by acidifying with trichloroacetic acid and then flushing with carbon dioxide for 5 min. Aliquots of the trichloroacetic acid supernatant were plated directly onto aluminum planchets, dried under an infrared lamp and the amount of ^{14}C fixed during the incubation measured.

In some experiments the dicarboxylic acid fraction of the incubation medium was isolated as described[1].

Fixation of inorganic phosphate was determined by measuring the incorporation of ^{32}P from labeled ortho-phosphate into the nucleotides. The nucleotides were isolated by absorption onto charcoal and eluted with 5% aqueous pyridine[8]. The pyridine eluents were plated onto pyrex planchets and dried under an infrared lamp. The relative counts from ^{32}P were determined after placing an empty aluminum planchet over the pyrex planchet containing the sample. This procedure blocked all of the counts from ^{14}C and measured only the counts from ^{32}P.

Hydroxamic acid derivatives of the acyl-ester compounds in the incubation medium were prepared as described by ARONOFF[9]. These derivatives were separated by paper chromatography using octyl alcohol–formic acid–water (3:1:3) as the solvent system[10]. Control and carrier derivatives were prepared from propionic anhydride and butyric anhydride. The spots on the paper were developed by spraying with ferric perchlorate solution. The area of the propionyl-hydroxamates was cut from the paper and counted.

RESULTS

Effect of butyrate on propionate metabolism by liver slices from non-fasted sheep

In the previous studies[1] it was found that butyrate exerted a marked stimulation on the metabolism of propionate from liver slices obtained from sheep starved 6–8 days. Preliminary work had revealed that this period of deprivation resulted in a drop in the liver carbohydrate from about 5 % to 1 %. Since the addition of glucose to the starved liver slices also increased the metabolism of propionate, it was of interest to ascertain whether the butyrate stimulatory effect would be found in tissues of non-fasted animals.

The results of adding butyrate as a cosubstrate to propionate with liver slices from a non-fasted sheep are shown in Table I. It is clear from these results that liver from fasted sheep is not required to show the stimulatory effect of butyrate on propionate metabolism. However, the magnitude of the stimulation that was obtained in this experiment was not as great as that obtained when tissue from fasted animals was used[1].

TABLE I

EFFECT OF BUTYRATE ON THE METABOLISM OF [1-^{14}C]PROPIONATE BY LIVER SLICES OF NON-FASTED SHEEP

All flasks contained 200 μmoles of propionate containing 1 μC carboxyl-labeled propionate. The control flasks contained an additional 200 μmoles of propionate and the cosubstrate flasks contained 100 μmoles butyrate. Incubation time, 3 h in Krebs–Ringer bicarbonate, pH 7 at 37°.

Fraction	*Cosubstrate* *None* *counts/min/flask*	 *Butyrate* *counts/min/flask*
CO_2	67,829	90,260
	68,280	100,913
Dicarboxylic acids	9,890	27,440
	10,610	21,300

Effects of butyrate on propionate metabolism by rat-liver slices

Since ruminant animals are unique in that they absorb large amounts of propionate, one might expect a species difference in the butyrate stimulatory effect. To test this hypothesis four experiments were conducted with rat-liver slices. In the first three of these experiments the rats were starved 24 h, and in the fourth the rat was fed *ad libitum*. The results of these experiments are shown in Table II. Although some stimulation of the formation of CO_2 from propionate was obtained in the first two experiments, none was obtained in the other two. Moreover, even in the first two experiments, butyrate did not increase the ^{14}C incorporation in the dicarboxylic acid fraction. Therefore, although a butyrate stimulation might occur in rat tissues, the stimulation was not as consistent nor as great as that obtained with sheep-liver slices.

Effects of butyrate on carbon dioxide fixation by propionate

In order to determine if butyrate stimulated propionate metabolism over the pathway involving the carboxylation of propionate[11] or via some other pathway,

TABLE II

EFFECT OF BUTYRATE ON THE METABOLISM OF [1-^{14}C]PROPIONATE BY RAT-LIVER SLICES

All flasks contained 3 g liver slices in Krebs–Ringer bicarbonate buffer pH 7.0 and 200 µmoles propionate containing 1 µC [1-^{14}C]propionate. The control flasks contained an additional 200 µmoles propionate and 100 µmoles butyrate was added to the cosubstrate flasks. All flasks were incubated at 37° for 3 h, except those from Expt. 3 which were incubated 1.5 h. The rat for Expt. 4 was not fasted.

Fraction	Expt. No.	Cosubstrate None counts/min/flask	 Butyrate counts/min/flask
CO_2	1	15,291	33,675
	2	213,333	376,470
	3	14,665	14,012
	4	20,616	24,348
Dicarboxylic acid	1	26,980	26,850
	2	12,490	16,440

an experiment was conducted using both ^{14}C labeled bicarbonate and [1-^{14}C]propionate.

The presence of butyrate stimulated the fixation of ^{14}C from labeled bicarbonate in addition to the incorporation of ^{14}C from [1-^{14}C]propionate into the dicarboxylic acid fraction (Table III). These findings coupled with the fact that the butyrate stimulatory effect is found irrespective of the metabolic fraction measured[1], strongly suggests that the site of stimulation must occur early in the propionate metabolic pathway. These findings also substantiate those of Black and Kleiber[12] and Pennington and Sutherland[13] who suggested that, in the ruminant, propionate was carboxylated to succinate.

TABLE III

EFFECT OF BUTYRATE ON THE FIXATION OF LABELED CARBON DIOXIDE IN THE DICARBOXYLIC ACID FRACTION BY LIVER SLICES FROM FASTED SHEEP.

Each flask contained 3 g liver slices in Krebs–Ringer bicarbonate pH 7.0 and 200 µmoles propionate. Incubation time was 3 h at 37°. Control flasks contained an additional 200 µmoles propionate and the cosubstrate flask, 100 µmoles butyrate. The labeled bicarbonate flasks contained 5 µC [^{14}C]$NaHCO_3$ and the labeled propionate flasks, 1 µC [1-^{14}C]propionate.

Cosubstrate	Labeled substrate HCO_3^- counts/min/flask	 Propionate counts/min/flask
None	7,320	4,052
Butyrate	19,360	16,646

Contribution of energy from cosubstrates in the presence of propionate

Friedberg *et al.*[14] obtained a stimulation by hydroxybutyrate on the carboxylation of propionate, but showed that the cosubstrate merely supplied the energy necessary for carboxylation of the propionate. It was doubted that a similar phenomenon could account for the butyrate stimulatory effect with sheep liver since citrate gave no stimulatory effect, yet was oxidized as rapidly as butyrate[1]. In order to further eliminate the energy hypothesis, two experiments were conducted in which the energy production by various substrates in the presence of propionate was determined. The energy production was estimated by measuring the amount of [^{32}P]ortho-phosphate fixed in the nucleotide fraction. In addition, β-hydroxybutyrate, succinate and α-ketoglutarate were tested for their capacity to stimulate propionate metabolism. The results of these experiments are shown in Table IV. Of the substrates tested, stimulation of propionate metabolism was obtained only with butyrate and β-hydroxybutyrate. No stimulation was obtained with α-ketoglutarate and an inhibition was obtained with citrate and succinate. In the previous studies[1] with liver from fasted animals, citrate neither stimulated nor inhibited propionate metabolism. It is conceivable that the explanation for the inhibition obtained in these experiments rests with use of tissue from non-fasted animals. It is interesting that adding citrate as a second cosubstrate abolished the stimulatory action of butyrate, and the corollary that butyrate did not overcome the inhibition of citrate.

The results of the determination of ^{32}P in the nucleotide fraction show that the compounds which failed to stimulate propionate metabolism, citrate, succinate and α-ketoglutarate, promoted, if anything, an increase in the amount of inorganic phosphate esterified. These findings fully substantiate the hypothesis that the mechanism of the butyrate stimulation is not simply an energy effect.

TABLE IV

EFFECT OF COSUBSTRATES ON THE METABOLISM OF PROPIONATE AND THE FIXATION OF INORGANIC PHOSPHATE

Each flask contained 3 g liver slices from non-fasted sheep in Krebs–Ringer bicarbonate and 200 μmoles propionate, 1 μC [1 ^{14}C]propionate and 1 μmole KH_2PO_4 containing 7 μC ^{32}P. The control flask contained an additional 200 μmoles propionate. The cosubstrates amounts were butyrate and β hydroxybutyrate, 100 μmoles; citrate, succinate and α-ketoglutarate, 200 μmoles. Incubation was at 37° for 1.5 h.

Cosubstrate	*Expt.*	*$^{14}CO_2$ counts/min/flask*	*^{32}P fixed counts/min/flask*
None	5	33,018	396
	6	23,676	382
Butyrate	5	44,264	394
	6	50,602	355
β-hydroxybutyrate	5	64,814	517
	6	78,765	246
Citrate	5	13,010	660
	6	8,455	457
Succinate	5	7,452	475
α-ketoglutarate	6	21,789	617
Butyrate + citrate	5	10,932	562
	6	5,587	369

Effect of isobutyrate, valerate, hexanoate and octanoate on propionate metabolism

Heretofore stimulation of propionate metabolism has been observed with

TABLE V

STIMULATION OF PROPIONATE METABOLISM BY MONOCARBOXYLIC ACIDS

All flasks contained 3 g liver slices in 20 ml Krebs–Ringer bicarbonate pH 7.0 and 200 μmoles propionate containing 1 μC [1 ^{14}C]propionate. All cosubstrates were added at a level of 100 μmoles. An additional 200 μmoles propionate was added to the control flask. Incubation was for 3 h at 37°. Incubation was not initiated until several hours after the animal was sacrificed.

Cosubstrate	*^{14}C in CO_2 counts/min/flask*
None	3,921
Butyrate	10,486
Isobutyrate	6,634
Valerate	10,663
Hexanoate	5,148
Octanoate	5,545

monocarboxylic acids, acetic and butyric, but not with polycarboxylic acids, citric, succinic and α-ketoglutaric. In order to ascertain if the stimulation would be found with other monocarboxylic acids, an experiment was conducted with liver slices from fasted sheep using as cosubstrates isobutyrate, valerate, hexanoate and octanoate. All of the cosubstrates tested stimulated the incorporation of ^{14}C from carboxyl-labeled propionate into carbon dioxide, but two degrees of stimulation are apparent (Table V). Isobutyrate, hexanoate and octanoate all stimulate to the same degree, but this is less than the stimulation obtained with valerate and butyrate. It is significant that both valerate and isobutyrate exert a stimulatory effect on propionate metabolism even though they are propionate precursors[15, 16].

Effect of butyrate on the metabolism of propionyl-CoA and on the metabolism of propionate plus CoA

The results of previous experiments[1] have suggested that the stimulatory effect of butyrate seems to be exerted at an early stage in the metabolism of propionate. In order to determine if butyrate stimulated the metabolism of propionate that had been

TABLE VI

EFFECT OF BUTYRATE ON THE FIXATION OF $^{14}CO_2$ IN THE PRESENCE OF PROPIONATE AND PROPIONYL-CoA

The enzyme system was extracted from sheep-liver acetone powder with 10 volumes of 0.1 *M* tris buffer, pH 8.0. The incubation mixture consisted of tris buffer, pH 8.0, 50 μmoles; $MgCl_2$, 5 μmoles; GSH, 4 μmoles; ATP, 4 μmoles; $KHCO_3$, 5 μmoles; $Na^{14}CO_3$, 3 μmoles, 0.9 μC; and the enzyme. All substrates were added in 0.5 μmole quantities. Incubations were carried out in 4-ml test tubes at 30° for 20 min under air. The reactions were stopped by adding 0.5 ml of 10% trichloroacetic acid. Values given are the mean of duplicate tubes except the tube with propionate and butyryl-CoA.

Additions	*^{14}C fixed counts/min/tube*
Propionate + CoA	3,753
Propionate + CoA + butyrate	5,798
Propionate + butyryl-CoA	6,749
Propionyl-CoA	8,704
Propionyl-CoA + butyrate	9,788
Butyryl-CoA	402

activated with coenzyme A, an experiment was conducted using a crude extract of sheep-liver acetone powder, the results of which are shown in Table VI. The fixation of ^{14}C by propionate from $^{14}CO_2$ was used as the criterion.

The presence of butyrate and butyryl-CoA increased the carboxylation of propionate 54% and 80%, respectively. Although replacing the propionate and coenzyme A with propionyl-CoA resulted in more than doubling the CO_2 fixation, the addition of butyrate to propionyl-CoA increased the CO_2 fixation only 12%. The small amount of ^{14}C fixed by the system in the presence of butyryl-CoA, but the absence of propionate provides evidence that the CO_2 fixation in the system employed was primarily the result of carboxylation of propionate. These results are suggestive that a transfer of coenzyme A may be involved in the stimulation of propionate metabolism by butyrate.

TABLE VII

EFFECT OF BUTYRATE AND BUTYRYL-CoA ON THE FORMATION OF PROPIONYL-CoA FROM [1-^{14}C]PROPIONATE

The enzyme system was an extract of sheep-liver acetone powder by 10 volumes of 0.1 *M* Tris buffer, pH 7.5. Incubation mixture contained 5 µmoles $MgCl_2$, 4 µmoles GSH, 4 µmoles ATP, 20 µmoles propionate containing 0.5 µC ^{14}C and the enzyme. Additions of 0.5 µmole CoA, butyrate or butyryl-CoA were made where indicated. Incubations were for 10 min at 30°. Reactions were stopped by adding 0.2 ml of 0.1 *M* hydroxylamine in ethanol.

Additions	*[^{14}C]Propionyl-CoA counts/min*
CoA	143
Butyrate + CoA	140
Butyryl-CoA	167

Transfer of coenzyme A.

In this experiment an attempt was made to obtain information on the transfer of CoA from butyryl-CoA to propionate. A Tris buffer extract of sheep-liver acetone powder and [1-^{14}C]propionate were incubated with and without butyryl-CoA. The hydroxamate derivative of propionyl-CoA was separated by chromatography and counted. The results are given in Table VII. Unfortunately, the results are inconclusive. The presence of butyryl-CoA resulted in a slight increase in the ^{14}C found in propionyl-CoA, but the presence of butyrate was without effect. The radioactivity recovered in the butyryl-CoA was negligible.

DISCUSSION

When one fatty acid is added to a system metabolizing another fatty acid, the usual effect observed is that of inhibition[2,5]. Thus the stimulation of propionate metabolism by monocarboxylic acids is somewhat unique. Although this stimulatory effect is not necessarily confined to starved ruminant liver, the magnitude of the stimulation is greater when starved sheep liver is used than when either rat liver or non-starved sheep liver is employed. Species difference with respect to the capacity for metabolism of volatile fatty acids has been reported by PENNINGTON AND APPLETON[5]. These investigators also reported[5] that the conversion of propionate to CO_2 was not altered by acetate. It is possible that the stimulatory effect was not observed because the animals used were not starved. Since the stimulatory effect is found with butyrate, isobutyrate, valerate, hexanoate, octanoate (Table V), β-hydroxybutyrate (Table IV) and acetate[1], but not with citrate, α-ketoglutarate or succinate (Table IV); it is apparent that the stimulatory effect on propionate metabolism is confined to the monocarboxylic acids.

The finding that butyrate stimulates the fixation of ^{14}C from $^{14}CO_2$ as well as from [1-^{14}C]propionate suggests that butyrate stimulates propionate metabolism over the pathway of carboxylation to succinate. Prospects of a condensation reaction between propionate and butyrate or between propionate and acetate are unlikely because propionate failed to stimulate the metabolisme of these compounds[1].

The evidence presented here indicates that metabolism of butyrate contributing the energy necessary for the carboxylation of propionate[14], thus stimulating its metabolism, is not adequate to explain the interrelationship between the two fatty acids. The butyrate stimulatory effect upon propionate was observed in liver slices in which a shortage of ATP would appear improbable. Furthermore, acids that failed to stimulate the metabolism of propionate were metabolized as rapidly as butyrate and fixed inorganic phosphate equally as well.

In view of the fact that butyrate stimulates the carboxylation of propionate and that the acids of the citric acid cycle are highly labeled[1], it is probable that butyrate exerts its influence early in the metabolism of propionate. The difference in the degree of butyrate stimulation on propionate plus CoA and propionyl-CoA (Table VI) is further proof of this hypothesis and indicates that the site of stimulation is prior to the formation of propionyl-CoA.

An hypothesis advanced to explain the butyrate stimulatory effect upon propionate metabolism can be based on the three following reactions:

$$\text{Propionate} + \text{ATP} + \text{CoA} \longrightarrow \text{Propionyl-CoA} + \text{AMP} + \text{PP} \qquad (1)$$

$$\text{Butyrate} + \text{ATP} + \text{CoA} \longrightarrow \text{Butyryl-CoA} + \text{AMP} + \text{PP} \qquad (2)$$

$$\text{Butyryl-CoA} + \text{propionate} \rightleftharpoons \text{Propionyl-CoA} + \text{butyrate} \qquad (3)$$

The first is the activation of propionate to form propionyl-CoA[11]. The second involves the activation of butyrate to form butyryl-CoA[17,18]. The third, a reaction not yet reported for animal tissues, involves the transfer of the coenzyme A moiety from butyryl-CoA to propionate. This last proposed reaction is similar to the transphorase reaction[19] in which coenzyme A is transferred from one fatty acid to another in *Clostridium kluyveri*. Since the enzyme systems that activate propionate show at best only marginal activity toward butyrate[20,21], and the butyrate activating enzyme is inactive toward propionate[17], it is possible that the activation of propionate could occur less readily than the activation of butyrate. Such effects could arise from differences in the rates of metabolism of the two enzyme systems and/or from the presence of a propionate specific deacylase. If one postulates that the activation of butyrate is more rapid than the activation of propionate, it is conceivable that a net reaction involving the activation of butyrate with coenzyme A and the transfer of coenzyme A from butyryl-CoA to propionate could occur more rapidly than the direct activation of propionate. There would be essentially no difference between the direct and indirect systems with respect to energy required for propionate activation.

Such an hypothesis is in accord with the findings in the studies of the stimulatory, effect of butyrate on propionate metabolism. It would be expected, as was observed, that butyrate would stimulate the fixation of CO_2 and propionate plus CoA but have no effect on propionyl-CoA. No stimulation was obtained with acids of the citric acid cycle (Table IV) as would be expected. In contrast, stimulation was found with the addition of β-hydroxybutyrate, isobutyrate, valerate, hexanoate and octanoate (Table V) and all of these acids are activated by the butyrate activating system[17]. Similarly, if the proposed transphorase does exist, it must be rather non-specific for the monocarboxylic acids from 3–8 carbon atoms.

Of course, this hypothesis would rest on much firmer ground if the existence of a CoA-transphorase could be established by isolation. The experiment on the isolation of [^{14}C]propionyl-CoA from the reaction of [1-^{14}C]propionate with butyryl-CoA although inconclusive is at least consistent with the existence of a CoA transphorase. Further work along these lines is in progress.

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Short Communications

Self-degradation of [^{60}Co]vitamin B_{12}

SMITH[1] has reported on the instability of vitamin B_{12} labeled with any of the radioactive cobalt isotopes. For some time we have been aware in our own studies with [^{60}Co]vitamin of the likelihood of self degradation. We have employed paper electrophoresis to study this problem and have reached independently the same conclusions as voiced by SMITH. We have also been able to identify the degradation products that are present in our samples.

Paper-electrophoretic data on our labeled vitamin B_{12} samples were determined at pH 6.2 using phosphate buffer at an ionic strength of 0.05. The concentration of the vitamin used was 10 μg/ml.

The electrophoretic separation was carried out in a horizontal paper electrophoresis unit (Reco), employing strips of Whatman No. 3 MM paper. A volume of 20 μl of the test solution was applied at the center. Separation was carried out for 6 h at a potential of 400 V.

The paper-electrophoretic strips were subjected to the autoradiographic procedure described by BARLOW AND FREDERICK[2]. To date five different lots (Merck) of [^{60}Co]-vitamin B_{12} have been studied. With time every sample has shown at least two radioactive peaks in addition to the unchanged vitamin. With the exception of vitamin B_{12} all peaks move toward the anode. A typical pattern is shown in Fig. 1.

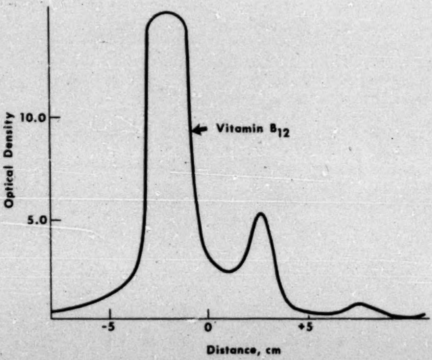

Fig. 1. Paper electrophoresis of a sample of [^{60}Co]vitamin B_{12} stored for at 4° 30 days after receipt.

Since excess cyanide was found to have no effect on our patterns, degradation of our [^{60}Co]vitamin B_{12} samples cannot be attributed to loss of cyanide with subsequent formation of aquocobalamin. A separate mobility check with a known sample of aquocobalamin definitely proved that it was not involved since its electrophoretic movement is toward the cathode.

The movement of the contaminants toward the anode suggested the possibility of deamidated derivatives. Subjecting a sample of cold vitamin B_{12} to mild acid hydrolysis as described by ARMITAGE *et al.*[3], we were able to reproduce the peaks found in the radioactive solutions, locating the areas in this case by their color. This suggests that the peaks in the radioactive vitamin B_{12} are mono- and dicarboxylic acid derivatives caused by the loss of one and two amide groups respectively.

TABLE I

STABILITY DATA

*Time (days)**	*Sample A-area (cm²)*		*Sample B-area (cm²)*	
	Peak 1	*Peak 2*	*Peak 1*	*Peak 2*
0	0.6	—	0.4	—
16	3.0	—	3.0	—
30	4.5	0.8	3.3	0.5
65	9.9	2.3	4.3	0.5

* After day of arrival.

By the use of preparative electrophoretic methods, the contaminants were isolated. The u.v. spectra of the two contaminants and of cold vitamin B_{12} were identical. Microbiological activity data showed the monocarboxylic acid to be about 1/3 as active as vitamin B_{12} itself and the dicarboxylic acid to be inactive.

Our available stability data indicates we are dealing primarily with a radiological effect. Quantitative figures for two samples are shown in Table I, in which the area of the new peaks due to degradation is tabulated as a function of time. A control sample of non-radioactive vitamin B_{12} showed no detectable change during this period of time.

Although the amount of the degradation product is small (5 %), it is significant in the analysis of vitamin-B_{12}-binding data obtained by electrophoretic methods. Since adequate controls have not been used in the past the validity of much of the published electrophoretic-binding data need be reexamined.

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Chemical detection of 3-indolylacetic acid in *Ustilago zeae* tumors*

Smut infections cause an increase in the auxin content of the host tissues[1]. This was demonstrated, by bioassay, in *Ustilago zeae* tumors, first by MOULTON[2], then by WOLF[3], VON GUTTENBERG AND STRUTZ[4] and HIRATA[5]. TURIAN'S observation[6] of high catalase levels in *U. zeae* tumors and the activation of acid phosphatase activity in tumor extracts[7] also supported the presence of a high auxin level[8]. However, upon chromatography[7] of an extract from small quantities of tumors, no IAA could be detected with SALKOWSKI[13] reagent.

These results, together with considerations of the absence or at the most a very low concentration of IAA in healthy corn seedlings[9,10], suggested that chemical detection of the pathological hyperauxiny in the tumorized tissues would require extraction of much larger amounts of tumor than previously used. Since the low concentration of IAA and the high concentration of interfering substances preclude direct chemical detection, it is necessary to use multi-step purification procedures. These procedures result in great loss of IAA during purification so a small amount of [2-^{14}C]IAA was added to permit correction for these losses. The radioactive IAA is also extremely useful in locating and following the IAA during the isolation.

Three main experiments were performed: the first using 1200 g of young tumors (mainly pre-chlamydospore stage), the second with 1500 g of maturing tumors (early chlamydospore stage), and the third with 1840 g of uninfected corn stalks (early tassel stage, collected at the same time as the younger tumors). The materials were collected in the field and stored at —20° until used. The data and the following technical description correspond to our isolation of IAA from younger tumors, and serve to illustrate the general methods used.

Tumors were ground in a Waring Blendor with sufficient 95 % ethanol to make the final alcohol concentration about 80 %. After filtration, 1.65 μg [2-^{14}C]IAA (specific activity $9.18 \cdot 10^4$ disintegrations/min/μg[11] or, under our counting conditions, 34,500 counts/min/μg), 7 g EDTA, and sufficient $NaHCO_3$ to make the ethanolic extract alkaline were added. The ethanol was removed *in vacuo* at 37° using a steam-driven vacuum pump connected to a "spray dry" apparatus, and the resulting 1.5 l extract further concentrated to about 700 ml with a rotating-film evaporator at 50° and 17 mm Hg. The concentrate (pH about 8.5) was filtered through glass wool, extracted with 2 volumes of peroxide-free diethyl ether and the ether discarded. The aqueous portion was adjusted to pH 3.5 with 10 N H_3PO_4 and extracted with 3 volumes ether. The combined ether fractions were concentrated *in vacuo* to about 100 ml, and extracted twice with 50 ml 8 % $NaHCO_3$. The combined bicarbonate extracts were adjusted to pH 4.0 with 10 N H_3PO_4 and extracted 3 times with equal volumes of ether. The combined ether extracts were dried over anhydrous Na_2SO_4 at 1°, and then concentrated *in vacuo* to a dark reddish-brown "oil". This residue was diluted to 20 ml with ether, and a 0.2-ml aliquot counted on an etched-glass planchet (2.69 cm^2 area) using a thin-window gas-flow Geiger-Müller tube. The total radioactivity in this fraction was 36,320 counts/min or about 64 % of the [2-^{14}C]IAA added.

The "oil" contains considerable acidic lipoidal material, possibly hydrolytic

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Abbreviations: IAA, indolylacetic acid; EDTA, ethylenediaminetetraacetic acid.

* Contribution No. 2574 from Michigan Agricultural Experimental Station.

products of the glucolipid ustilagic acid[12]. In an effort to separate IAA from this lipoidal material, the "oil" was partitioned 2 times (after removing the ether) between 5 ml acetonitrile and 5 ml ligroin. The ligroin layers (light yellow) were discarded (less than 200 counts, about 0.2 % of the IAA, is lost with each extraction). After removal of the acetonitrile *in vacuo*, column chromatography was attempted using 25 g of diatomaceous silica (analytical grade Celite, Johns-Manville Co., U.S.A.) moistened with 20 ml 0.1 M phosphate, pH 6.50, as the stationary phase, and ether saturated with the same buffer as the mobile phase. A total of 26 fractions of 3.5 ml were collected and radioactivity was estimated by counting 0.2-ml aliquots from each tube. Fractions 6–18 were combined, dried over anhydrous Na_2SO_4, and the ether then removed *in vacuo*. The residues were paper chromatographed for 14 h on Whatman 3 mm paper (which had previously been washed with alkaline EDTA and 0.1 N HCl) in isopropanol–ammonia–water (8:1:1) as the ascending solvent. The IAA runs just above, but does not separate completely from a dark-brown pigment, so this entire area was eluted with 50 % ethanol. The eluted sample was rechromatographed as above on a 6-inch strip but with a slightly more aqueous solvent (7:2:1). Upon examination of the chromatogram under u.v. light and monitoring for radioactivity, it appeared that some separation of IAA from the pigment had been achieved so that they could be eluted separately. After elution and counting as above, it was found that the dark-brown pigmented fraction contained 8,350 counts/min while the light-yellow fraction contained 22,780 counts/min. Thus at this stage total IAA recovery was about 55 %. Since 73 % of this recovered IAA was in the light-yellow fraction, this was further purified by paper electrophoresis at 1° on Whatman 3 mm paper (washed as above) using 0.1 M citrate buffer, pH 5.25. The mobility of IAA under these conditions, as judged by the application of known IAA to one edge of the electrophoretogram, is $2.94 \cdot 10^{-5}$ $cm^2/V \cdot sec$. After drying, the IAA was located by means of u.v. light, by radioactivity, and by spraying a narrow test strip containing known IAA with Ehrlich's reagent (1 % *p*-dimethylaminobenzaldehyde plus 8.5 % HCl in 95 % ethanol). The pigment-free IAA area was eluted with 50 % ethanol, and the elution volume adjusted to 1.5 ml. Measurements of the recovery of radioactivity on duplicate 0.05-ml samples indicated a recovery of 10,737 counts/min, or 18.8 % of the [2-^{14}C]IAA added. For the quantitative estimation of the IAA recovered, a 0.3-ml sample was assayed with 1.2 ml of SALKOWSKI reagent[13] using the Klett photoelectric-colorimeter with micro-cells, and a No. 54 filter. The rate of color development was followed at 10-min intervals for 40 min, and was about the same in both standards and sample. The IAA concentration was 4.0 μg/0.3 ml. Thus a total of 20 μg of IAA was isolated. Since about 0.3 μg of this IAA was the [2-^{14}C]IAA added, the total non-radioactive IAA isolated would be 19.7 μg, and the total IAA present in the tumor tissue was 105 μg.

The results of the described isolation, together with isolations from older tumors and corn stalks, are summarized in Table I. The general methods used in the latter isolations were the same as those described for younger tumors, with the specific differences indicated in the footnotes.

The values obtained should be considered roughly quantitative, being limited by the colorimetric methods used. It should be noted that the partition column was not a satisfactory purification step in this study, though it has been employed satisfactorily in the case of corn seedlings[9]. It appeared that the proper partition was

TABLE I

THE INDOLYLACETIC ACID CONTENT OF *Ustilago zeae* TUMORS AND CORN STALKS

Tissue	*Fresh wt.* (g)	*IAA isolated** μg	*Recovery of* [$2\text{-}^{14}C$]*IAA* %	*IAA present corrected for recovery*	
				μg/kg fresh wt.	*M*
Younger tumors** (mainly pre-chlamydospore)	1200	19.70	18.8	87.5	$5.0 \cdot 10^{-7}$
Maturing tumors*** (early-chlamydospore)	1500	5.85	18.5	21.0	$1.2 \cdot 10^{-7}$
Corn stalks§ (early-tassel)	1840	1.10	14.3	4.2	$2.4 \cdot 10^{-8}$

* Corrected for recovery of [$2\text{-}^{14}C$]IAA. Amount added was: younger tumors, 1.65 μg; maturing tumors, 0.825 μg and corn stalks 3.30 μg.

** See text.

*** After column chromatography, one half the combined IAA fraction was paper chromatographed in isopropanol–ammonia–water (8:1:1), and *n*-butanol–ammonia–water (10:1:1) successively. The eluent from the latter paper chromatogram was recombined with the remaining (one-half) IAA fraction from the column. The combined fractions were again column chromatographed, paper chromatographed as above (isopropanol–ammonia–water), and electrophoresed.

§ After column and paper chromatography (isopropanol–ammonia–water), the IAA-containing fraction was electrophoresed twice. The IAA concentration was estimated in this case on the final electrophoretogram by spraying it with Ehrlich's reagent and comparing it with IAA standards. Recovery of [$2\text{-}^{14}C$]IAA was estimated just before the final electrophoresis on duplicate aliquots, and a radioautogram indicated that all the radioactivity on the final electrophoretogram was IAA.

prevented by the oil-like residue obtained upon concentrating the acid ether fractions.

Ustilago zeae tumors are characterized by an IAA level much above that of the uninfected corn stalk tissue. Younger tumors had about 20 times, and older sporulating tumors about 5 times the $2.4 \cdot 10^{-8}$ *M* IAA concentration found in uninfected stalks.

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Rapid increase of rat-liver triglycerides following carbon tetrachloride poisoning

Detailed chemical studies of the relative quantities of different lipid fractions in the liver following CCl_4 poisoning have not been carried out, although histological evidence has suggested that the increased lipid is largely neutral lipid. Since triglycerides account for only 5–10 % of total liver lipid, it appeared possible that an analysis of the liver for triglycerides, following CCl_4 poisoning, might reveal large changes in this fraction heretofore obscured by measurement of total-lipid content. The results fully confirmed the expectation.

Male rats, 168–206 g (Holtzman Company, Madison) previously fasted 16 h, were force fed a CCl_4–mineral oil mixture (1:1) under light ether anaesthesia. The mixture was administered at a dose of 0.5 ml/100 g body weight. Anaesthetized control animals received only mineral oil. 1 h and 3 h later the rats were killed by cervical section, bled, and the livers removed and weighed. A sample of liver, 1–1.5 g, was extracted for total lipid in chloroform–methanol (2:1) according to Folch *et al.*[1]. Phospholipids were separated from the non-phospholipids by chromatography on silicic acid. Triglycerides were determined by glycerol analysis[2] after saponification of the non-phospholipid fraction. The significance of the difference between means was estimated with "students" t-test.

TABLE I

INCREASE IN LIVER TRIGLYCERIDES, TOTAL LIVER LIPID, AND LIVER WEIGHT FOLLOWING CCl_4 POISONING

	No. of rats	*Liver weight, g/100 g body wt.*	*Total liver lipid, mg/100 g body wt.*	*Total liver triglycerides mg/100 g body wt.*
1 h post CCl_4				
Control	11	3.04 ± 0.036*	151.5 ± 2.57	14.3 ± 1.61
CCl_4-fed	13	3.03 ± 0.052	160.7 ± 1.96	19.1 ± 0.48
Increase		No change	6.1 %	33.6 %
Significance		$P > 0.5$	$P < 0.01$	$P < 0.01$
3 h post CCl_4				
Control	6	3.06 ± 0.045	151.7 (144–155)**	14.6 (12.1–17.7)
CCl_4-fed	6	3.25 ± 0.071	200.0 (172–239)	43.1 (34.4–53.5)
Increase		6.2 %	31.6 %	195 %
Significance		$P < 0.025$	***	***

* Data given as mean ± standard error, except where the range is indicated.
** Mean plus range.
*** The significance of the change is beyond doubt, since the control and CCl_4-fed rats form two distinct groups with no overlap, as indicated by the ranges.

The data (Table I) show that liver triglycerides increase rapidly during the initial period of intoxication. The increase in liver triglycerides is a better index of early changes in hepatic-lipid content than the increase in total liver lipids. The liver weight shows even less change than total lipids. These data are significant since they clearly establish the very early rise in liver-triglyceride content.

Oberling and Rouiller[3], in a study with the electron microscope, observed pathological alterations in the ultrastructure of the hepatic parenchyma, especially in the endoplasmic reticulum, within several hours following intraperitoneal injection of CCl_4 to rats. Glucose-6-phosphatase activity of rat liver is reduced to approx. 50 % of control levels 4 h following CCl_4 feeding (R. O. RECKNAGEL, unpublished). These observations, coupled with the early rise in hepatic triglyceride content, show that the onset of pathological changes following CCl_4 administration is rapid. It appears evident that in order to reveal the primary biochemical lesion, future studies must be concerned with events occurring early in the course of the developing pathology.

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Thiols of low molecular weight in *Hevea brasiliensis* latex

The strong reducing power of fresh latex has been attributed by earlier workers[1] to "non-enzymic" sulphydryl materials of unidentified composition. The presence of such factors is of particular relevance to the problem of rubber biosynthesis since it has recently been shown that sulphydryl co-enzymes are essential for sterol[2] and other isoprenoid syntheses via "isopentenol PP isomerase"[3].

In the present study samples of fresh latex serum were obtained from Malaya in the form of centrifuged serum solids (freeze dried) as described in an earlier communication[4] and extracted in dilute H_2SO_4 at pH 2.5. Aliquots of the soluble fraction were examined for –SH content by the colorimetric method recently proposed[5,6]. The oxidised sulphydryl content was determined by repeating the assay after complete reduction by zinc and HCl. "Pure" cysteine similarly reduced was used as a calibration substance for this procedure and extinction measurements made at 540 mμ in a Hilger spectrophotometer.

The values obtained for a number of latex-serum samples are shown in Table I from which it can be seen that the total sulphydryl content, after reduction of oxidised material, is remarkably constant from one sample to another, the samples being obtained from bulked latex collected during three successive months. Approx. 10 % of this total –SH is present in the oxidised form. Recent (unpublished) investigations in this laboratory on small samples of fresh latex taken from young *Hevea* plants are in close agreement with these results.

Abbreviations: GSH, reduced glutathione; CSH, cysteine.

The quantitative separation of the thiols from other materials present was next achieved by extraction of the latex-serum preparation with dilute H_2SO_4 (generally at pH 2.5 and 0°), precipitation with $HgSO_4$ in the presence of lead acetate, treatment with gaseous H_2S and removal of excess gas in the presence of N-ethylmaleimide[7] at pH 6–8. Separation of the individual sulphydryls and their identification was carried out on measured amounts of the concentrated solution by paper chromatography in 80 % isopropanol, the –SH compounds being detected on the paper by treatment with anhydrous alcohol–KOH[8]. Quantitative assessment of the spots was then made by spraying chromatograms in an atmosphere of CO_2 with ninhydrin–acetone solution[9], eluting in 50 % ethanol and estimating in a Hilger absorptiometer with filter No. OGRI. Fig. 1 is a photograph, taken through an Ilford tri-colour green

TABLE I

TOTAL "–SH" CONTENT OF LATEX SERUM

Sample No.		*μequiv. –SH/g centrifuged dried solids*				
		Original	*Mean*		*Reduced*	*Mean*
72	(i)	17.35	16.73	(i)	20.60	20.60
	(ii)	16.10		(ii)	20.60	
73	(i)	20.40	19.33	(i)	20.40	20.20
	(ii)	18.25		(ii)	20.00	
74	(i)	18.40	17.78	(i)	19.22	19.17
	(ii)	17.16		(ii)	19.12	
Average			17.95			19.99

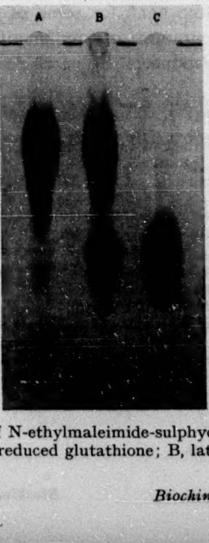

Fig. 1. The transient pink spots of N-ethylmaleimide-sulphydryl complexes in a chromatogram treated with anhydrous alkali. A, reduced glutathione; B, latex-serum concentrate; C, cysteine.

filter, of the pink spots on a typical chromatogram developed with alkali. This shows clearly that practically all the –SH content extracted in the above manner is located in positions corresponding closely to those of GSH and CSH markers.

Table II summarises the results of quantitative measurements of several such chromatograms and indicates the reproducible nature of the GSH values and the variable values for CSH. Nevertheless the combined average total –SH content of 16.59 μmoles/g isolated in this way accords well with the total equivalent –SH content, prior to zinc reduction, of 17.95 μequiv. (Table I) and indicates that at least 93 % of the total –SH in latex is in the form of glutathione and cysteine in the average molal ratio of 1.6.

TABLE II

GLUTATHIONE AND CYSTEINE CONTENT OF SERUM SOLIDS

Sample No.	*Extraction procedure*		*Number of extractions*	*Average μmoles/g centrifuged dried solids*	
	Temperature	*pH*		*GSH*	*CSH*
72	0°	2.5	5	11.40	11.59
	0°	4.5	3	10.10	6.62
	100°	2.5	3	17.60	4.96
73	0°	2.5	2	10.75	3.31
	0°	4.5	2	9.13	6.62
74	0°	2.5	2	10.10	3.31
Average values obtained by cold extraction					
μmole/g solids				10.30	6.29
μmole/ml original serum				0.72	0.44

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The action of mitochondrial preparations on glycolysis

During the last few years interest has been shown in studying the effects of mitochondrial preparations on glycolytic systems with a view to elucidating the control mechanisms exerted by each system on the other. In particular Aisenberg, Reinfarje and Potter[1] and Aisenberg[2] have described a Pasteur effect by freshly prepared rat-liver mitochondria on a glycolytic system prepared from brain. Sissakian and Pinus[3] also studied the effect of rat-liver mitochondria on glycolysis but used a muscle supernatant as the source of glycolytic activity. An important discrepancy between the results from the two groups of workers is that whereas Aisenberg *et al.* found that liver mitochondria inhibited glycolysis under aerobic conditions only, Sissakian and Pinus found inhibition under both aerobic and anaerobic conditions.

Experiments described in this report show that rat-liver mitochondria were found to inhibit the anaerobic glycolysis of a rat-brain supernatant.

For the most part the studies by the groups of workers cited above together with those of Von Korff[4], and Gatt and Racker[5] involved the non-biological system of recombining mitochondria from one tissue with a glycolytic system either prepared from a different tissue or reconstructed from isolated enzymes. In the few recorded experiments in which mitochondria were added to a glycolytic system prepared from the same tissue the mitochondria did not inhibit glycolysis (muscle [3]; Walker 256 carcinoma[1]; ascites tumour[6]). The present report describes experiments in which brain mitochondria were added to a brain supernatant glycolytic system and in no instance was inhibition of glycolysis observed.

The results given in Table I show that in an atmosphere of 95 % N_2 + 5 % CO_2 freshly prepared rat-liver mitochondria brought about a marked inhibition of the rate of glycolysis of brain supernatant. The amounts of mitochondria added were similar to those used by Aisenberg *et al.*, as was the quantity of brain supernatant. Since inhibition of anaerobic glycolysis occurred on adding liver mitochondria under the present experimental conditions the results are inconsistant with the view that liver mitochondria exert a Pasteur effect on brain supernatant glycolysis. Under

TABLE I

THE EFFECT OF FRESHLY PREPARED BRAIN AND LIVER MITOCHONDRIA ON ANAEROBIC GLYCOLYSIS OF BRAIN SUPERNATANT

Glycolytic activity of a rat-brain supernatant[1] was measured by the evolution of CO_2 from a medium containing 0.0015 M DPN, 0.04 M nicotinamide, 0.0055 M $MgCl_2$, 0.0011 M ATP, 0.0045 M phosphate buffer, pH 7.4, 0.025 M $KHCO_3$ and 0.011 M glucose in a total volume of 2.2 ml. Incubation was at 37° in an atmosphere of 95 % N_2 + 5 % CO_2. Mitochondria[7] in 0.25 M sucrose were added to the above mixture as indicated.

Mitochondria	*Original weight of tissue (mg)*	*µl CO_2 evolved between 5 and 20 min*		
		Mitochondria alone	*Supernatant alone*	*Mitochondria + Supernatant*
Liver	875	13	114	21
	525	31	114	65
	175	42	114	162
Brain	1080	23	92	413
	648	23	92	344

identical experimental conditions brain mitochondria stimulated the glycolytic rate of brain supernatant (Table I). The addition of freshly prepared brain mitochondria, equivalent to 600 mg original weight of tissue, to brain supernatant in an atmosphere of 95 % O_2 + 5 % CO_2 doubled the rate of glycolysis.

The effects of liver and brain mitochondria on anaerobic glycolysis remained unchanged after the mitochondria had been stored at + 2° in 0.25 *M* sucrose for 48 h.

From these results it seemed unlikely that oxidative mechanisms, either alone or coupled to phosphorylation, were involved. Acetone-dried powders of brain and liver mitochondria were added to the glycolytic system (Table II). It was found that acetone-dried brain mitochondria stimulated glycolysis several fold. Similarly prepared liver mitochondria had less effect on glycolysis. Measurements were made of the uptake of inorganic phosphate from the medium. On addition of acetone-dried mitochondria the changes in uptake of inorganic phosphate were equivalent to the changes in the output of CO_2. If the output of CO_2 is expressed as μmoles of lactic acid and the disappearance of inorganic phosphate as μmoles esterfied the ratio of inorganic phosphate to lactic acid remained approx. 1 (Table II).

TABLE II

THE EFFECT OF ACETONE-DRIED LIVER AND BRAIN MITOCHONDRIA ON GLYCOLYSIS

The μl of CO_2 evolved has been corrected for the amount evolved in the absence of added substrate. The μmoles of lactic acid have been calculated from the amount of CO_2 evolved, assuming that this output was due solely to lactic acid formation.

	Acetone powder (mg)	*μl CO_2 evolved between 0 and 25 min*	*Lactic acid (μmoles)*	*Inorganic phosphate disappearance (μmoles)*	*Inorganic phosphate/lactic acid*
Brain supernatant alone	0	85	3.8	3.2	0.85
Plus acetone-dried brain mitochondria	1	110	4.9	5.35	1.09
	2	128	5.7	6.84	1.2
	3	158	7.05	8.7	1.23
	5	245*	—	—	—
Plus acetone-dried liver mitochondria	0	82.5	3.68	3.86	1.05
	2	91.5	4.07	4.0	0.98
	4	100	4.45	5.03	1.13
	6	94.5	4.2	4.7	1.12
	10	67.5	3.01	3.06	1.02

* This value was extrapolated from a 10-min value of 98 μl CO_2.

Acetone-dried brain and liver mitochondria brought about the release of 3 μmoles phosphate from 1 μmole ATP. It has previously been shown that ATPase activity can influence the rate of glycolysis[5,8]. The results plotted in Fig. 1 show that amounts of acetone-dried brain and liver mitochondria having identical phosphatase activity do not have an identical effect on the rate of glycolysis. Brain mitochondria stimulated glycolysis over the whole range of phosphatase activity used while liver mitochondria first stimulated then inhibited glycolysis. The results with acetone-dried liver mitochondria resemble those described by GATT AND RACKER using potato apyrase.

As originally shown by CRANE AND SOLS[9], brain mitochondria contain a high hexokinase activity. The addition of a yeast-hexokinase preparation provoked a large stimulation of the rate of glycolysis of brain supernatant (Table III). When glucose was replaced by glucose 6-phosphate as substrate neither yeast hexokinase, acetone-dried nor fresh brain mitochondria increased the output of CO_2 above that of the control.

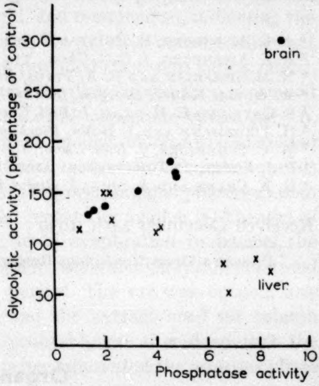

Fig. 1. The relationship between phosphatase activity of acetone-dried mitochondria and their effect on glycolysis. The phosphatase activity is expressed as µmoles inorganic phosphate liberated from ATP in 25 min at 37°. The effect on glycolysis is expressed as the percentage activity of brain supernatant in the presence of added mitochondria when the control activity is taken as 100 %.

TABLE III

EFFECT OF HEXOKINASE AND OF FRESH AND ACETONE-DRIED BRAIN MITOCHONDRIA ON GLYCOLYSIS WITH GLUCOSE 6-PHOSPHATE AS SUBSTRATE

Expt. 2 was under aerobic conditions. The values given for brain supernatant plus fresh brain mitochondria have been corrected for the CO_2 evolved by mitochondria without supernatant. The fresh brain mitochondria were from 440 mg original wet weight of brain.

Expt.		*µl CO_2 evolved between 0 and 15 min*	
		Glucose	*Glucose 6-phosphate*
1	Brain supernatant alone	48	100
	+ hexokinase (5 units)	125	86
	+ acetone-dried brain mitochondria (4 mg)	150	88
2	Brain supernatant alone	69	143
	+ fresh brain mitochondria	166	157

The soluble fraction of rat-brain homogenate although able to carry out glycolysis is limited at the hexokinase level. The large stimulation of glycolysis brought about by the addition of brain mitochondria, whether fresh, aged or acetone-dried could be explained by the presence of hexokinase in the mitochondrial preparations.

Whether the location of over 60 % of the total hexokinase activity in the mitochondria plays a predominant role in the control of glycolysis in intact brain cells remains a disputable point, but the overwhelming influence that this enzyme exerts on the rate of glycolysis of the soluble fraction of brain homogenates in *in vitro* studies might tend to mask the influence of other control mechanisms.

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Organic dialysis of lipids

Chloroform–methanol extracts of tissues contain both relatively simple lipids (triglycerides, lecithins and cephalins, cholesterol esters, etc.) and more complex, larger lipids (cerebrosides, gangliosides, proteolipids, mucolipids, etc.). In the course of chromatographing such extracts from erythrocytes ghosts[1] it became evident that large amounts of various simple lipids were present. To remove these small compounds and thus improve the chromatography of the complex lipids dialysis of the chloroform–methanol extract against organic solvents was tried and found to be successful. Furthermore the partition-dialysis procedure of FOLCH *et al.*[2], in which chloroform–methanol extracts are dialyzed against water to remove non-lipid contaminants, was also carried out. Both these dialysis procedures were applied to extracts of erythrocyte ghosts prepared as described below.

Whole human blood was centrifuged and the buffy layer and supernatant discarded. The cells were washed at least twice with isotonic saline, after which they were osmotically ruptured by suspension in 6 volumes of distilled water. Centrifugation at 3900 × *g* for 30 min produced a clear deep-red supernatant and a deep-red sediment. These sedimented ghosts were washed repeatedly with distilled water and centrifuged until the supernatant was colorless (6–8 washings), at which time the ghosts were a light pink (due to a small amount of hemoglobin tightly bound to the membrane or internal structures[3]). Extraction of the ghosts with chloroform–methanol was carried out by dispersing approximately 1 vol. (400 ml) of the packed ghosts in 1 vol. 0.1 *M* phosphate buffer, pH 7.0 (to control the pH and quantitate the lipid extraction[4]) and adding 6 vol. chloroform–methanol (2:1) while the solution was rapidly stirred. Absence of the aqueous phase resulted in the formation by the ghosts of large dark-brown rubbery clumps of material (presumably indicating denaturation) which were extremely difficult to manipulate. After rapid stirring for 30 min at room temperature the solution was centrifuged and the clear, lower chloroform layer removed, followed by a similar re-extraction of the aqueous layer containing the ghost residues. The chloroform extracts were combined, evaporated nearly to dryness under reduced pressure, and taken up in 50 ml chloroform–methanol (2:1).

Wetted cellulose* dialysis sacs were half filled with the chloroform–methanol extract and then twice dialyzed, with gentle stirring, against 2 l chloroform–methanol at 2° for 24 h. The sacs filled rapidly, became turgid, and remained so, indicating the presence of non-dialyzable lipid solutes on the inside. The cellulose became somewhat hard and brittle, but the sacs did not break or leak provided they were kept straight. To remove aqueous solutes and to wash the extract[2] the dialysis was then continued at 2° for 24 h against two changes of 2 l each of 0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl and previously equilibrated against chloroform–methanol (2:1)[4]. The sacs were then opened, the contents centrifuged, and the upper aqueous phase, presumably containing strandin[2], discarded. The lower, organic phase was then replaced in new, wetted cellulose bags and dialyzed repeatedly against 2-l changes of chloroform–methanol (2:1) at 2° for 24 h until, upon evaporation to dryness, the outside fluid was free of solutes, indicating the complete removal of dialyzable material (8 changes were required to reach this stage of purity). The sac was opened, any sediment that had formed was centrifuged off and the extract used for column chromatography[1]. The dialysis sequence might profitably be altered so that the initial dialysis is against water or buffer followed (after removal of the aqueous phase inside the sac) by the organic dialyses.

Control experiments have shown that cholesterol, cholesterol palmitate, tripalmitin, triolein, and dipalmitoyl phosphatidyl choline are completely dialyzable under these conditions. Thus it appears that material remaining inside the dialysis sac has a higher molecular weight, or is more complex, than these simple lipids. This organic dialysis procedure provides a method of selectively removing small lipid-soluble compounds quantitatively under mild conditions. Dialysis against other lipid solvents, such as diethyl ether, benzene, etc. should be equally feasible unless, of course, the integrity of the cellulose tubing is destroyed. It is likely that during the exposure to chloroform–methanol the pores in the cellulose enlarge, but apparently not sufficiently to permit passage of the large molecules present in the extract. Finally, organic dialysis should provide a simple means for gradually altering the composition of lipid solvents and thus allowing the selective precipitation and separation of closely similar dissolved lipids.

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Preliminary Notes

A device for obtaining cell membranes

The conventional methods of tissue disruption such as homogenization, ultra-sonic vibration, freezing and thawing, osmotic shock, compression and decompression, Mickle glass-bead vibrator, and enzymic digestion either destroy the membrane in the process of liberating the other cellular components or do not disrupt the cell. For muscle tissue, the failure of these methods is not surprising, in view of the 100–200 Å thickness of the muscle cell membrane and the close proximity of the large, highly viscid myofibrils.

The technique and apparatus briefly described here was developed and shows promise as a relatively gentle means of obtaining the membranes of certain tissue cells. This new method and its applications will be described more fully elsewhere.

The principle of the method is to submit the tissue cells to simultaneous squeezing and shearing forces. A loose suspension of cells is placed within the free end of a long piece of dialysis tubing (Visking size 36/32) which has been previously washed thoroughly, dried in an inflated state, and then deflated and rolled up smoothly onto a suitable spool. The free end of the tubing is fed between the rollers of the apparatus described below. As the tubing is passed between the rollers, cells are also dragged through and submitted to pressures and shear forces. The contents of liver and adipose cells are largely squeezed out, while muscle cell fragments are spread out into sheets. The tubing is subsequently collected in a beaker containing saline, and when thoroughly moistened additional saline is passed through the tubing to wash out the contents. Conventional cell-fractionation procedures may then be used to purify the separated cellular components. Little difficulty is experienced in identifying by light microscope the membrane fraction of liver and adipose tissue. The muscle-fibre fragments, however, are reduced to sheets of membranous structureless material containing membrane as well as compressed myofibrils. A means of identifying the true muscle membrane is now being sought.

The apparatus used to date (see Fig. 1) contains two non-laminated, cylindrical Tufnol rods (supplied by Tufnol, Ltd.) 2.5 cm in diameter and 9.65 cm long, and carefully selected for their trueness. To reduce flexibility, the rods are pierced down their centres with stainless-steel pins 1 cm in diameter. The accuracy with which the pins are placed within the Tufnol rods is crucial to the efficiency of the apparatus. Experience has shown that Tufnol is a better material than either Perspex or stainless steel, probably because its degree of hardness is between these latter two materials. The ends of one rod (M) are machined down by 0.1 cm and fitted with 0.5 cm wide stainless-steel tires (T) of surface just below the remainder of the unmachined rod. Bull races (R) are tapped onto the ends of the steel pins of the untyred rod (F). The rods or rollers are mounted horizontally and in parallel into an appropriately slotted, horizontal open box frame of 1/4 inch brass. The frame is slotted in such a way that the roller (F) is fixed by its bull races, whereas the roller (M) may be moved either toward or away from (F). Handles fastened to the ends of the steel pins protruding beyond the frame allow the rollers to be rotated. The device used to push the movable roller (M) against the fixed roller (F) consists of an 8.4 × 1.3 × 3.8 cm brass plate (P) with two 2 cm in diameter, 0.5 cm wide stainless-steel wheels (W) at either end, mounted above one another in such a manner that when the brass plate is placed within the box frame and adjacent to roller (M), the wheels (W) engage the tyres (T) without allowing contact between the roller and the plate. Two brass bars (B) are attached to the plate and, after piercing the box frame through two carefully placed holes, are joined together by another brass plate. Two clamps (C) draw this second brass plate towards the end of the box frame, creating a force which pushes the movable roller (M) onto the fixed roller (F). The entire assembly is suspended from two stands by means of two additional brass bars protruding from the far end of the frame. Above the assembly is placed the spool (S) of dialysis tubing, and below is placed a beaker for collecting the tubing after its passage between the rollers.

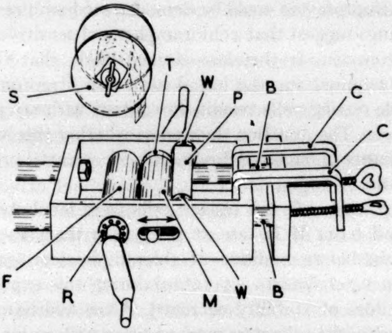

Fig. 1. Apparatus for obtaining cell membranes.

The tissue-cell suspensions used in this apparatus have been prepared by brief Waring Blendor homogenizations in large volumes of buffered saline, followed by straining through Terylene mesh (size dependent upon the type of tissue) to remove connective tissue and undisrupted tissue. The filtrate is subsequently subjected to numerous washings and brief centrifugations in order to separate the more slowly sedimenting cellular contents from the intact cells. Yields of intact cells are low but ample. An initial perfusion of the intact organ *in situ* removes the blood cells and considerably simplifies the task of obtaining a pure suspension of the cells desired. Other methods of preparing cell suspensions from intact tissue should be adequate.

Further refinements of the above procedures are under development and will be reported at a later date.

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Chloramphenicol-sensitive and -insensitive phases of the lethal action of streptomycin

Earlier experiments[1] have shown that exposure of resting cells of *Escherichia coli* B to streptomycin (10 µg/ml) causes only a minor decrease in viable count for about an hour. After this prior exposure, further incubation in the presence of streptomycin results in a logarithmic decline in viability.

The present experiments were designed to determine the nature of the process which occurs during the prior exposure to streptomycin before loss of viability occurs, and the relationship of this process to subsequent death of the cells. A means to differentiate the two phases was suggested by the findings of ANAND AND DAVIS[2] that chloramphenicol blocks the lethal effect of streptomycin in a growing culture. Preliminary experiments showed that a similar chloramphenicol-blocking effect on the lethal action of streptomycin could be demonstrated with resting-cell suspensions of *E. coli*. These findings suggest that a chloramphenicol-sensitive synthesis is required for killing by streptomycin. It therefore seemed likely that this chloramphenicol-sensitive synthesis, required for the lethal action of streptomycin, occurs during the interval in which resting cells remain in contact with streptomycin before loss of viability commences. The question then arose whether this streptomycin-induced reaction is itself the cause of bacterial death, or is an essential precursor for the lethal action of the antibiotic.

To decide this question, *E. coli* resting cells were incubated in a solution containing 1 % NaCl and 0.011 *M* lactate at pH 7.0, in the presence of streptomycin (10 µg/ml), for 60 min before addition of chloramphenicol (30 µg/ml). The viable count decreased from $1.7 \cdot 10^7$/ml to $4.5 \cdot 10^6$/ml during this exposure. In the absence of streptomycin, no loss of viability occurred. After addition of chloramphenicol to stop further synthesis, the cells were washed twice with water containing 30 µg/ml of chloramphenicol to remove residual streptomycin and then resuspended in the NaCl-lactate solution containing 30 µg/ml chloramphenicol and varying concentrations of streptomycin, as indicated in Table I. Cells incubated without streptomycin and then exposed to chloramphenicol for 2 h showed little or no loss of viability.

Addition of chloramphenicol before prior exposure to streptomycin prevents killing. When the cells are exposed to streptomycin for 60 min before addition of

TABLE I

EFFECT OF PRIOR EXPOSURE TO STREPTOMYCIN ON SUBSEQUENT KILLING OF *Escherichia coli*

Viable count/ml in presence of streptomycin (10 µg/ml)		*Viable count after 2 additional hours in chloramphenicol plus streptomycin*			
		Concentration of streptomycin			
Zero time	*After 60 min*	*Zero*	*10 µg/ml*	*20 µg/ml*	*40 µg/ml*
$1.7 \cdot 10^7$	$4.5 \cdot 10^6$	$2.2 \cdot 10^6$	$6.7 \cdot 10^5$	$2.8 \cdot 10^4$	$8.7 \cdot 10^3$

Resting cells of *E. coli* B were suspended in 1 % NaCl and 0.011 *M* lactate at pH 7.0. The first 60-min incubation was carried out in the presence of 10 µg/ml streptomycin. Chloramphenicol (30 µg/ml) was then added, and the cells were washed twice by centrifugation, and then resuspended in the NaCl-lactate medium containing 30 µg/ml chloramphenicol and the indicated concentrations of streptomycin for 2 additional hours. Viable counts were made by surface plating in quadruplicate on nutrient agar.

chloramphenicol, the rate of killing is a function of the external streptomycin concentration. Prior exposure to streptomycin, then, confers an increased sensitivity to the lethal action of streptomycin in the presence of chloramphenicol.

ANAND *et al.*[2,3] have proposed that exposure to streptomycin results in synthesis of cellular membrane with impaired permeability characteristics, and that this impaired permeability causes eventual death of the cell. Under such an hypothesis, the external concentration of streptomycin in the presence of chloramphenicol should have had no effect on viability. Actually, as seen from Table I, the number of survivors was inversely proportional to the external streptomycin concentration in the presence of chloramphenicol when the cells had been previously exposed to streptomycin.

It would therefore seem that the lethal effect of streptomycin is exerted in two phases: (1) a preparatory phase which is markedly less lethal and can be blocked by chloramphenicol followed by (2) a more direct lethal phase which is insensitive to chloramphenicol.

It is attractive to consider the preparatory phase as an induction process in view of its sensitivity to chloramphenicol. The specificity of such an induction by streptomycin has not, of course, been proven by the present experiment, but further work on this aspect is in progress.

The induction process might involve synthesis of a protein which would serve to activate streptomycin for its lethal action, or might involve the synthesis of a permease without which streptomycin could not accumulate within the cell in sufficient concentration for lethal action.

The latter possibility seems more amenable to test of the two, especially since ANAND, DAVIS AND ARMITAGE[3] have shown that chloramphenicol blocks accumulation of [^{14}C]streptomycin in sensitive bacteria and that resistant bacteria do not accumulate streptomycin even in the absence of chloramphenicol. From this it can be further postulated that mutation to resistance may involve loss of ability to synthesize the permease.

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The enzymic conversion of threonine to aminoacetone

It was shown by ELLIOTT[1–3] that when *Staphylococcus aureus* is incubated aerobically in a medium containing either glycine and glucose or threonine, aminoacetone accumulated in the medium. When *Rhodopseudomonas spheroides* was used, an aminoketone, which was identified as aminoacetone, accumulated in the medium when the organisms were incubated either aerobically in the dark, or anaerobically in the light, with either glycine plus α-oxoglutarate and fumarate or threonine as substrates[4].

The present communication is concerned with the action of an enzyme which has been found in cell-free extracts of *Rps. spheroides* which catalyses the following reaction.

$$\text{Threonine} + \text{DPN}^+ \longrightarrow \text{Aminoacetone} + \text{DPNH} + \text{H}^+ + \text{CO}_2$$

This enzyme reaction has not been described before, although the probability of threonine being a precursor of aminoacetone is strongly indicated by the results of ELLIOTT[1–3] with whole cells.

Rps. spheroides was grown anaerobically in the light according to LASCELLES[5]. The organisms were harvested, washed, and a thick suspension made in 0.05 *M* phosphate buffer, pH 7.4. The cells were then disrupted in the Hughes press[6]. The resulting suspension was centrifuged at 105,000 × *g* for 120 min. The clear pink supernatant, after dialysis against phosphate buffer at 4° overnight, was used as the enzyme solution.

The assay system, total volume 0.75 ml, contains L-threonine (50 μmoles), DPN$^+$ (0.5 μmole), enzyme solution (equivalent to 1 mg protein) in 0.1 *M* Tris, pH 8.8. Incubation was at 37° for 1 h. Aminoacetone was estimated at the end of the reaction on an aliquot of the trichloroacetic acid supernatant by condensation with acetylacetone according to MAUZERALL AND GRANICK[7]. Under the conditions described generally about 0.09 μmole/h of aminoacetone were synthesised.

The enzyme is specific for DPN$^+$, no aminoacetone being formed in its absence or when TPN$^+$ is used. Addition of pyridoxal phosphate to the dialysed enzyme extract does not increase the activity.

The pH optimum was found to be 8.8 when using 0.1 *M* Tris. If the reaction is done in 0.1 *M* carbonate–bicarbonate buffer, pH 8.8, the activity is reduced by 50 %.

By following the reaction at 340 mμ and estimating the aminoacetone formed, it was shown that for every mole of aminoacetone formed, a mole of DPN$^+$ is reduced.

The enzyme is active with L-threonine and D-allothreonine, the two isomers having the same configuration on the hydroxyl-bearing carbon atom. The other two isomers, D-threonine and L-allothreonine, are not attacked and have no inhibitory action. DL-Serine is not acted on, nor does it inhibit.

There are two possible reaction mechanisms (see also ELLIOTT[3]),

$$\text{I} \quad \underset{\text{threonine}}{\text{CH}_3\text{CH(OH)CHNH}_2\text{COOH}} \xrightarrow{\text{DPN}^+} \underset{\alpha\text{-amino-}\beta\text{-oxobutyric acid}}{\text{CH}_3\text{COCH(NH}_2\text{)COOH}} \xrightarrow{-\text{CO}_2} \underset{\text{aminoacetone}}{\text{CH}_3\text{COCH}_2\text{NH}_2}$$

$$\text{II} \quad \underset{\text{threonine}}{\text{CH}_3\text{CH(OH)CHNH}_2\text{COOH}} \xrightarrow{-\text{CO}_2} \underset{\text{1-aminopropan-2-ol}}{\text{CH}_3\text{CH(OH)CH}_2\text{NH}_2} \xrightarrow{\text{DPN}^+} \underset{\text{aminoacetone}}{\text{CH}_3\text{COCH}_2\text{NH}_2}$$

Abbreviations: DPN$^+$, oxidised diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN$^+$, oxidised triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)-aminomethane.

DL-1-Aminopropan-2-ol, the D-isomer of which is the postulated intermediate in pathway II, was tested with the enzyme system. No aminoacetone was formed at a variety of pH values with DPN$^+$ or TPN$^+$, nor did it inhibit the conversion of L-threonine to aminoacetone. This shows that the reaction goes according to pathway I.

LAVER *et al.*[8] have shown that α-amino-β-oxobutyric acid decarboxylates spontaneously under physiological conditions, having a half-life of much less than 1 min at pH 7. Consequently, no separate enzyme need be postulated for the decarboxylation step. The possibility remains that the decarboxylation occurs simultaneously with the dehydrogenation on the enzyme surface. Evidence has been presented for a decarboxylation of L-threonine to D-1-aminopropan-2-ol, labelling being found specifically in the 1-aminopropan-2-ol fragment in Vitamin B_{12} when it is biosynthesised in the presence of [^{15}N]threonine[9]. This result could also be explained by formation of aminoacetone from threonine by the enzyme described in this communication, followed by its reduction to 1-aminopropan-2-ol.

The enzyme has been purified 6-fold, and the purified fraction has the same properties as those described above for the crude extract.

This enzyme reaction probably occurs in a large number of tissues and may be of general importance in metabolism. Biosynthesis of aminoacetone by cell-free extracts from glycine and acetyl coenzyme A has been reported[10, 11]. Aminoacetone is synthesised in relatively large amounts under certain conditions by *Staph. aureus*[1–3] and by *Rps. spheroides*[4]; it has been identified as a constituent of the antibiotic micrococcin P[12], and a compound having similar properties has been described in human urine[7]. ELLIOTT[13] has shown that aminoacetone is oxidised to methylglyoxal by an enzyme in ox plasma and SHEMIN[14] has reported that in rats administered aminoacetone labelled with ^{14}C in the carbon atom bearing the amino group, the label appears in CO_2, formate, and in the ureido groups of uric acid.

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Rat-liver carbamyl transferases and biotin

In cell-free enzyme preparations from *Streptococcus lactis*[1] and *Lactobacillus arabinosus*[2] biotin deficiency was shown to reduce markedly the carbamylation of ornithine to citrulline and of aspartate to N-carbamylaspartate, indicating a role of the vitamin in these reactions. In the present study we investigated the possible role of biotin in animal transcarbamylation reactions.

Rats were maintained on a biotin-deficient ration containing 20 % spray-dried raw egg white. Control animals received intramuscularly two doses of 100 µg biotin per week per rat. The experimental group came down with a severe deficiency in 6–7 weeks (normal average weight 269 g, deficient 145 g).

Citrulline formation in normal and biotin-deficient preparations of liver mitochondria is shown in Table I. Contrary to the findings with bacterial enzymes[1,2], no significant difference in transcarbamylase activity could be observed between the normal and deficient groups. A similar experiment with another batch of rats, 4 in each group, also gave no significant difference (normal 123 ± 23, deficient 101 ± 16 µg citrulline/0.2 mg protein). The mitochondrial preparation when preincubated with a large excess of avidin for 2 h at room temperature also had no effect on the enzyme activity. Similarly, 400 units[3] of avidin did not inhibit the activity of the extract of acetone powder of mitochondria.

TABLE I

ORNITHINE CARBAMYL TRANSFERASE ACTIVITY OF NORMAL AND BIOTIN-DEFICIENT RAT-LIVER MITOCHONDRIA

The complete system contained: 33 µmoles tris(hydroxymethyl)aminomethane buffer pH 7.4, 5 µmoles $MgCl_2$, 26 µmoles carbamyl phosphate, 20 µmoles ornithine and 0.2 mg mitochondrial enzyme protein[8]; final volume was 1 ml and the reaction mixture was incubated for 15 min at 38°. Citrulline was determined according to ARCHIBALD[9], protein by the method of LOWRY *et al.*[10] and biotin according to SEMENZA *et al.*[11] Enzyme activity was assayed in duplicate at three levels.

Biotin (mµg/g fresh liver)		*µg Citrulline formed*	
Normal	*Deficient*	*Normal*	*Deficient*
1013	146	240	205
971	249	160	150
1106	264	160	180
1467	225	163	195

We have also investigated[4] the effect of biotin deficiency on aspartate carbamyl transferase in rat liver. In the first series of experiments, 5 control rats, receiving biotin as before, formed an average of 39 ± 10 µg carbamylaspartate/mg protein compared to 35 ± 6 µg by the deficient animals. In view of the observation of MACLEOD *et al.*[5] regarding the effect of time of injection of biotin on citrulline synthesis, we included two additional control groups in the second series. The experimental details and the results are given in Table II. Again no significant difference could be observed between the various groups. Similarly, preincubation of the enzyme with avidin did not change the specific activity of the preparation.

These data on the liver enzymes extend and support our previous observations[4]. Very recent evidence[6] confirms our findings and suggests that the observed effect

TABLE II

ASPARTATE CARBAMYL TRANSFERASE ACTIVITY OF NORMAL AND BIOTIN-DEFICIENT RAT LIVER

The complete system contained: 100 μmoles diethanolamine-HCl buffer pH 9.2, 10 μmoles L-aspartate, 20 μmoles carbamyl phosphate and 2–5 mg enzyme protein (12,000 × *g*, 45-min supernatant of 10 % liver homogenate in isotonic KCl containing 0.01 *M* ethylenediaminetetra-acetate, pH 7.8). Enzyme activity was assayed in duplicates at two or three levels. Final volume was 1 ml and the reaction mixture was incubated for 15 min at 38°. The reaction was stopped with 1.5 ml 0.5 *M* $HClO_4$, the supernatant after centrifugation was passed through a column of Dowex-50 and carbamyl aspartate was determined according to KORITZ AND COHEN[12].

	μg Carbamylaspartate/mg protein			
	*Normal**	*Normal***	*Normal****	*Deficient*
	43	55	48	34
	60	66	52	46
	40	48	48	65
	47	49	50	62
	32	36	49	—
Average	44 ± 10	51 ± 8	49 ± 2	52 ± 8

* Stock diet; received biotin injection 48, 24 and 4 h before the experiment.
** Egg-white diet plus biotin injection twice a week; also received biotin injections as above.
*** Egg-white diet plus biotin injection twice a week.

of biotin on the bacterial ornithine carbamyl transferase is indirect. In view of these results, the original observation of FELDOTT AND LARDY[7] that the vitamin is in some way involved in the synthesis of carbamyl phosphate seems pertinent. However, it is possible that this effect is also indirect.

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Zur Frage der Beziehungen zwischen Mahler-Enzym und Diaphorase zur mitochondrialen Cytochrom *c*-Reduktase; Ablösung, Eigenschaften und Reinigung der mitochondrialen DPNH-Cytochrom *c*-Reduktase*

1952 beschrieben EDELHOCH und Mitarbeiter[1, 2] die Ablösung einer DPNH-Cytochrom *c*-Reduktase aus Mitochondrien mittels 10 % Alkohol bei 42°. Von TSOU UND WU[3] wurden auf Grund von unterschiedlicher pH-Abhängigkeit, Substrat-Affinität und der Tatsache, dass aus der BAL-gehemmten, partikelgebundenen Reduktase ein wirksames Enzym nach MAHLER extrahiert werden konnte, Zweifel an der Nativität des Mahler'schen Enzyms erhoben.

Aus eigenen Untersuchungen können weitere Aussagen gemacht werden, die zur Schlussfolgerung führen, dass das Mahler'sche Enzym ein Kunstprodukt ist. Zwischen diesem Enzym und den artifiziell aus der nativen Cytochrom-*c*-Reduktase herrührenden Diaphorasen bestehen enge Wechselbeziehungen. Diese Schlüsse sind unter anderem durch folgende Beobachtungen gestützt:

1. In früheren Untersuchungen[4] war nachgewiesen worden, dass aus mitochondrialer Cytochrom *c*-Reduktase bei Inkubation unterhalb pH 6.5 über die Stufe einer mitochondrialen Diaphorase eine lösliche Diaphorase entsteht. Es handelt sich um einen von der Histidin-Ionisation abhängigen Prozess.

2. Rinderherz-Mitochondrien, deren Reduktaseaktivität durch Inkubation bei pH 6.0 zum grössten Teil in mitochondriale Diaphorase umgewandelt worden war, was sich in der Abnahme von Reduktaseaktivität widerspiegelt, liefern dieselbe Ausbeute an Mahler-Enzym wie native Mitochondrien.

3. Durch Inkubation mit Gift von *Naja naja oxiana* (siehe auch ref. 5), lässt sich aus Rinderherz-Mitochondrien lösliche Diaphorase gewinnen. Aus Tabelle I ist zu ersehen, dass die Ausbeute an Mahler-Enzym und Diaphorase in einem wechselseitigen Verhältnis stehen. Wird vorerst mit *Naja naja* Diaphorase abgelöst, so sinkt die nach Mahler extrahierte Diaphorase-Aktivität auf etwa 60 %; die umgekehrte Reihenfolge bringt ein Absinken der mit *Naja naja* extrahierten Aktivität auf 40 % mit sich. Die Summe der Aktivitäten nach beiden Extraktionsmethoden ist annähernd konstant. Dieser Versuch darf als entscheidender Beweis für die gemeinsame Quelle beider Enzymaktivitäten gewertet werden.

TABELLE I

ABLÖSUNG DER DIAPHORASEN VOM MITOCHONDRIEN-STROMA

	Reihenfolge		*Aktivität*		
Versuch	*Naja naja*	*Alkohol*	*Naja naia*	*Alkohol*	*Summe*
1	1	2	9.5	7.0	16.5
2	2	1	3.8	12.0	15.8

Es wurde lösliche Diaphorase-Aktivität aus Mitochondrien durch Inkubation mit *Naja naja* 1 Stunde 37° pH 7.4 und Alkohol-Extraktion der Mitochondrien 15 Min 42° gewonnen. Diaphorase mit Dichlorphenolindophenol in 0.03 *M* Phosphat-Puffer pH 7.4 gemessen. Eine Einheit = µval reduzierter Farbstoff/Min/ml Enzym-Lösung. Die Reihenfolge nennt die Behandlungsfolge der Mitochondrien, ob zuerst mit *Naja naja* inkubiert und dann extrahiert wurde oder umgekehrt.

Abkürzung: BAL, British Anti-Lewisite (2,3-Dimercaptopropanol).

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4. Es ist gelungen, durch kombinierte Extraktion mit *Naja naja* und Butanol Cytochrom *c*-Reduktase aus Rattenleber-Mitochondrien zu gewinnen, die in ihrer pH-Abhängigkeit und Substrat-Affinität dem nativen Enzym der Mitochondrien entspricht. Tabelle II zeigt einen Reinigungsgang.

TABELLE II

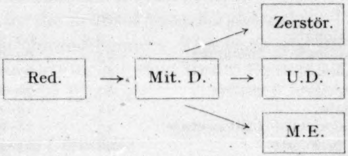
REINIGUNG DER DPNH-CYTOCHROM *c*-REDUKTASE AUS RATTENLEBER-MITOCHONDRIEN

	Reduktase E/ml	*Eiweiss mg/ml*	*Spez. Akt. E/mg*	*Gesamt-Akt.*
Ausgang	3,04	9·35	0,33	177
Butanol-Extrakt	1,61	1,66	0,97	101
Calzium-Gel	8,05	0,51	15,8	28

Lebermitochondrien werden mit Schlangengift 3 Stunden 30° inkubiert, die Partikel abzentrifugiert und mit Butanol-Alkohol pH 8.9 extrahiert, abgelöste Reduktase an Calzium-Phosphat-Gel adsorbiert und mit Phosphat-Puffer pH 6.1 eluiert.

Aus den Beobachtungen lassen sich auch vorläufige Schlüsse auf Struktur und Halterung der Enzyme ableiten. Aus dem Umstand, dass die Ablösung der Diaphorase sowohl durch Ionisation der Imidazolgruppe als auch durch *Naja naja*-Phospholipase bewirkt wird, lässt sich vermuten, dass sowohl Imidazol-bedingte Wasserstoff-Bindungen als auch hydrophobe Bindungen die Cytochrom *c*-Reduktase an die Mitochondrien-Struktur haltern. Die Tatsache, dass das Mahler-Enzym nach vorangegangener Inaktivierung der mitochondrialen Cytochrom *c*-Reduktase aus gebundener Mitochondrien-Diaphorase entsteht, lässt vermuten, dass seine artifizielle Bildung durch eine Umlagerung unter Rekombination mit Eisen sekundär zustande kommt. Nachfolgendes Schema gibt die genetischen Beziehungen zwischen Mitochondrien-Reduktase und ihren Folgeprodukten wieder. Ausführliche Mitteilungen über diese Befunde erfolgen an anderer Stelle (*Acta Biol. et Med. Germ.*).

Schema der Reduktase-Umwandlungen.



Red. = mit. Reduktase.
Mit.D. = mitochondrialgebundene Diaphorase.
U.D. = abgelöste Diaphorase.
M.E. = Mahler'sches Enzym.

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The oxidation of glutamate by rat-heart sarcosomes

The oxidation of glutamate by isolated mitochondria is generally believed to proceed via glutamic dehydrogenase, followed by oxidation of the α-ketoglutarate by the Krebs cycle. In the course of recent experiments on the mechanism of oxidative phosphorylation[1, 2] we have found that only a small fraction of the glutamate oxidized by rat-heart sarcosomes follows this pathway. Most is oxidized to aspartate, according to reactions (1) and (2), the latter being that part of the Krebs cycle lying between α-ketoglutarate and oxaloacetate.

$$\text{Glutamate} + \text{Oxaloacetate} \longrightarrow \alpha\text{-Ketoglutarate} + \text{Aspartate} \qquad (1)$$

$$\alpha\text{-Ketoglutarate} + {}^{3}/_{2}\,O_2 \longrightarrow \text{Oxaloacetate} + H_2O + CO_2 \qquad (2)$$

$$\textit{Sum} \quad \text{Glutamate} + {}^{3}/_{2}\,O_2 \xrightarrow{\text{(Oxaloacetate)}} \text{Aspartate} + CO_2 + H_2O \qquad (3)$$

That a product of the oxidation of succinate was necessary for the oxidation of glutamate was shown by the fact that the oxidation was inhibited 96.6 % by 0.02 M malonate, and that this inhibition was largely relieved by the addition of fumarate or malate (Table I). Pyruvate was ineffective while oxaloacetate gave variable results, probably because added oxaloacetate is very rapidly decarboxylated by heart sarcosomes. The glutamic dehydrogenase activity of swollen or sonicated sarcosomes was found to be very low. This is in agreement with results of VON EULER *et al.*[4] with whole heart extracts and with the very slow rate of the KREBS–COHEN[5] dismutation of α-ketoglutarate (in the presence of NH_3) by rat-heart sarcosomes[6].

TABLE I

EFFECT OF MALONATE AND KREBS-CYCLE INTERMEDIATES ON GLUTAMATE OXIDATION BY RAT-HEART SARCOSOMES[3]

*Substrate(s) and inhibitor***	*Rate of O_2 uptake (glutamate alone = 100)**		
	Mean	*Range*	*No. of expts.*
Glutamate	100		12
Glutamate + malonate	3.4	1.5–4.4	12
Glutamate + malonate + fumarate	75	63–85	4
Fumarate + malonate	20	17–24	3
Glutamate + malonate + malate	81	70–89	4
Malate + malonate	21	18–26	3
Glutamate + malonate + oxaloacetate	45	28–61	5
Oxaloacetate + malonate	38	23–47	4
Glutamate + malonate + pyruvate	9	4–14	2
Pyruvate + malonate	5	1–10	2

* The O_2 uptake was measured in a medium similar to that given in the legend to Fig. 1 of ref. 1; 0.02 M inorganic phosphate was present in all experiments, and the final pH was 7.5.

** Final concentrations: glutamate, 5–20 mM; oxaloacetate, 20–50 mM; other substrates and malonate, 20 mM.

Paper chromatography[7] showed that during glutamate oxidation aspartate —and no other amino acid—was formed. The aspartate formation was completely blocked by malonate and restored by the addition of malate or oxaloacetate. Under anaerobic conditions only oxaloacetate addition gave rise to aspartate. Preliminary experiments with the aspartic decarboxylase of *Nocardia globerula* described by

CRAWFORD[8] confirm that aspartate is formed during glutamate oxidation, in amounts close to that predicted by reaction (3) (see Table II).

These observations show that oxidation to aspartate is the most important pathway for glutamate oxidation by isolated rat-heart sarcosomes. It seems likely that oxaloacetate is the acceptor of the amino group in reaction (1), and that the reaction is catalysed by glutamic–aspartic transaminase, present in rat-heart sarcosomes[6]. Since this enzyme is known to be present also in mitochondrial preparations of other tissues, it is to be expected that reaction (3) is not restricted to rat-heart sarcosomes. Indeed we have found that aspartate is formed during the oxidation of glutamate by rat-liver mitochondria and Ehrlich ascites-tumour cell mitochondria.

TABLE II

ASPARTATE FORMATION DURING GLUTAMATE OXIDATION BY RAT-HEART SARCOSOMES

Reaction time (min)	*O_2 uptake (μatoms)*	*Aspartate formation** (μmoles)*	*ΔO/ΔAsp*
4	3.39*	1.23	2.8
8	6.74	1.79	3.8
12	9.24	2.47	3.7
16	12.37	3.60	3.4
20	15.02	3.89	3.9

* Mean of interpolated values in other flasks.

** Measured by the method of CRAWFORD[8] using differential manometers (gas volume, 6–7 ml).

It is clear that the glutamate–aspartate conversion should be kept in mind when glutamate is used as the substrate in studying mitochondrial oxidations. Moreover, the pathway of glutamate oxidation described here may be responsible for the rapid oxidation of glutamate in tissues with a low level of glutamic dehydrogenase, as proposed for brain by STRECKER[9].

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Two modes of lysine synthesis among lower fungi: evolutionary significance

Recently, lysine biosynthesis has recommended itself as a seemingly reliable indicator of certain phylogenetic relationships[1-3]. In these studies, various diagnostic ^{14}C tracers, including aspartic acids, alanine, and acetate, have proved useful. All the eubacteria, pseudomonads, and actinomycetes examined appear to synthesize lysine in one general manner (presumably via α,ε-diaminopimelic acid), and all the ascomycetous and basidiomycetous fungi in another (presumably via α-aminoadipic acid)[1-3]. Higher (vascular) plants show labeling patterns of the bacterial type[3], as do the green algae tested[2]; *Euglena*, however, resembles the higher fungi[2]. The present communication reports findings with lower fungi, an assemblage of organisms which have long attracted students of evolution.

Table I shows the labeling pattern found for one member of the Saprolegniales, *Achlya bisexualis* (strain C6[4], kindly furnished by Dr. ALMA W. BARKSDALE), and for one of the mucorales, *Rhizopus stolonifer* (ATCC 10404). In the case of the latter organism, the 3-carbon of aspartic acid is appreciably incorporated into protein lysine, whereas the 4-carbon of aspartic acid and the 1-carbon of alanine are not; the 2-carbon of acetate labels lysine intensely (in comparison with the labeling of protein aspartic acid and threonine). Closely parallel results were obtained with *Mucor hiemalis* (ATCC 1205), and *Cunninghamella blakesleeana* (ATCC 8688a). The pattern of these mucorales is essentially the same as that of the higher fungi and *Euglena*[2,3] and suggests the α-aminoadipic acid-lysine path. In the case of *A. bisexualis*, both the 3- and 4-labeled aspartic acids contribute substantially to protein lysine, and the 2-labeled acetate makes a roughly equivalent contribution; the 1-carbon of alanine labels lysine more heavily than can be accounted for by the conversion of the alanine to aspartic acid. The same labeling pattern was displayed by *A. americana*

TABLE I

INCORPORATION OF TRACERS INTO PROTEIN AMINO ACIDS
(AS SPECIFIC ACTIVITY RELATIVE TO THE RESPECTIVE PROTEIN ASPARTIC ACID VALUES)

Organism	*Tracer*	*Aspartic acid*	*Threonine*	*Lysine*
Achlya bisexualis	DL-[3-^{14}C]Aspartate	100	109	139
	DL-[4-^{14}C]Aspartate	100	122	130
	DL-[1-^{14}C]Alanine	100	92	205
	[2-^{14}C]Acetate	100	123	136
Rhizopus stolonifer	DL-[3-^{14}C]Aspartate	100	107	63
	DL-[4-^{14}C]Aspartate	100	105	< 5
	DL-[1-^{14}C]Alanine	100	114	< 5
	[2-^{14}C]Acetate	100	111	1060

The organisms were cultivated in 20 ml medium in 250-ml flasks at 28°, *Achlya*, without shaking, in a glucose–glutamate–cystine–salts medium similar to liquid M-medium[4] and *Rhizopus*, with shaking, in a glucose–salts medium (Medium S, H. J. VOGEL, unpublished). Approximately 2–5 mg (20–40 μC) of tracer were supplied per flask. For the determination of relative molar amounts of the three protein amino acids, experiments with uniformly labeled major carbon sources were performed. The cultures obtained were subjected to the harvesting, extraction, hydrolysis, paper chromatography, and counting procedures previously described, and specific activities were computed as before[3,5].

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(ATCC 10977), *Saprolegnia ferax* (ATCC 10396), and *S. parasitica* (ATCC 11393), grown on a chemically defined medium[6] at 23°. The behavior of these saprolegniales resembles that of the green algae and bacteria (as well as the vascular plants)[1-3] and points to the α,ε-diaminopimelic acid-lysine path. (Details of the two lysine paths have been reviewed elsewhere[7]; *cf.* GILVARG[8].)

In preliminary experiments, a representative of the blastocladiales, *Allomyces macrogynus* (strain Burma 1Da[9], obtained through the courtesy of Dr. RALPH EMERSON and grown on Medium B[10]), exhibited a labeling pattern like that of the mucorales and higher fungi.

From the standpoint of evolution, it is of particular interest that, in regard to lysine synthesis, the saprolegniales studied resemble the green algae and bacteria, but differ from the other phycomycetes examined and from the higher fungi. The present results, in the context of the earlier ones[1-3], support the view that the two types of lysine path represent biochemical organelles of ancient origin and are useful characters in the exploration of phylogenetic links.

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Movements of fluid and glucose in an everted sac preparation of rat colonic mucosa

We have examined the capacity of everted sacs of rat colonic mucosa to transport fluid and glucose *in vitro*. The muscular coats were removed to reduce hindrances to fluid and solute movements across the submucosal tissues and the hydrostatic pressure across the sac wall was maintained constant during the experiment by the use of a method similar to that described by CRANE AND WILSON[1].

A piece of glass tubing, 7.5 mm in diameter, with a constriction at one end and tapered at the other, forms a cannula. This is cooled (4°) and a piece of rat descending

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colon, previously rinsed with ice-cold oxygenated saline is pulled over it. The colon, so distended, is then stripped by making a longitudinal incision with a blunt scalpel; the divided muscle layer retracts and can be peeled off with the fingers, leaving behind the mucosa, supported by a thin layer of muscularis mucosae. The lower end of the colon is tied at the constriction in the cannula and the remainder gently "milked" over the ligature and tied at the other end to give an everted sac suspended from the cannula.

Sacs, thus prepared, were immersed in Krebs bicarbonate medium, containing penicillin (20 units/ml) and streptomycin (40 units/ml), gassed at 38° with 95 % O_2 and 5 % CO_2. Into the interior of the sac, the "serosal side", was placed 0.5–1 ml Krebs medium. Water uptake from the mucosal fluid was measured by weighing the sac and cannula at intervals. Alternatively, the fluid "transport", i.e. that appearing on the serosal side, was estimated by collection and weighing. "Uptake" and "Transport" can thus be estimated independently, and they are found to be closely related, as shown for example in Table I.

TABLE I

EFFECT OF OSMOTIC GRADIENTS ON FLUID ABSORPTION

Segments of rat descending colon, length about 3 cm containing 30 mg fat-free dry weight, incubated 2 h in Krebs bicarbonate medium, gassed with 95 % O_2, 5 % CO_2; 28 m*M* glucose present on serosal side only. Initial osmotic activity on serosal side about 300 mOs*M*. Osmotic gradient altered by changing NaCl concentration on mucosal side. ΔP: difference in osmotic activity, mucosal–serosal.

Mucosal fluid	*ΔP (mOsM)*		*Fluid uptake from mucosal fluid mg/mg/h**	*Fluid transport into serosal fluid mg/mg/h**	*Solute transport into serosal side μOsm/mg/h*
	Initial	*Final*			
"Hypertonic"	85	57	0.30 ± 0.37 (4)	—0.97 ± 0.29 (4)	1.77 ± 0.16 (4)
"Hypertonic"	48	26	3.19 ± 0.27 (8)	1.35 ± 0.22 (8)	2.07 ± 0.15 (8)
"Isotonic"	— 3	—17	8.15 ± 0.63 (7)	6.37 ± 0.62 (5)	2.73 ± 0.21 (5)
"Hypotonic"	—49	—48	11.20 ± 1.44 (7)	9.65 ± 1.32 (7)	2.67 ± 0.39 (7)

* Values are means ± standard error of mean (number of experiments). Weight of tissue are fat-free dry wt.

With glucose, 12 m*M*, present initially on both sides, there was a loss of glucose (141 ± 14 (8)) m*M*/kg/h* from the mucosal fluid as well as from the serosal fluid (104 ± 3 (16) m*M*/kg/h) when measured over a 2-h period. There is thus no evidence for active transport of glucose inwards against a concentration gradient. Lactate production in the presence of glucose was found to be 100–170 m*M*/kg/h. in agreement with the findings of DICKENS AND WEIL-MALHERBE[2], and to be compared with values equivalent to 600–1,000 m*M*/kg/h for the aerobic lactate production by rat small intestine[2,3,4]. In the presence of N_2 and 5 % CO_2, lactate production by the colon increased to 800–1,000 m*M*/kg/h.

Unlike the small intestine, where water absorption proceeds only in the presence of glucose[5,6], it was found that the colon maintains a limited rate of fluid uptake for the first 2 h in the absence of added substrate (6.1 ± 0.7 (13) mg fluid/mg/h, compared with 12.1 ± 0.7 (18) mg/h with 12 m*M* glucose on both sides). With 10 m*M* acetate on both sides, the fluid uptake was 10.9 ± 0.8 (4) mg/mg/h and similar evidence for the enhancement of fluid uptake was obtained with *n*-butyrate, acetoacetate and pyruvate. Fluid uptake was also increased in the presence of D(+)-mannose but not in that of D(+)-galactose. Under anaerobic conditions (gas mixture: 95 % N_2 + 5 % CO_2) in the presence of glucose, fluid uptake was reduced to 0.5 ± 0.1 (4) mg/mg/h.

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* Mean ± standard error of mean (no. of experiments), expressed as m*M* glucose/kg fat-free dry wt./h.

In Table I are shown the fluid uptake and transport, and total solute transport, with differing osmotic gradients across the sac wall. A modified cannula was used so that the serosal volume was increased to about 5 ml and the changes in the serosal fluid osmotic pressure, due to solute movement, minimised. The serosal fluid was mixed by a slow stream of gas bubbles. It can be seen that the rate of fluid absorption decreased with increasingly adverse osmotic gradients. Nevertheless, against an adverse osmotic gradient which decreased during absorption from 48 milliosmolal to 26, there was a significant inward net transport of fluid ($P < 0.001$), thus demonstrating that, like the rat small intestine[7], rat colon *in vitro* is capable of inducing an active fluid transfer.

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Complex formation between adenine oligonucleotides and polyuridylic acid

The formation of complexes between long-chain polyribonucleotides has been described for several homopolymer pairs: poly (A + U)[1], poly (I + C)[2], and poly (A + I)[3]. The influence of chain length (*n*) on such interactions has not been assessed, although WARNER AND BRESLOW[4] have reported that poly U containing as few as 24 nucleotide units will interact with poly A of $n > 160$. The present paper presents evidence for complex formation between poly U and adenine ribooligonucleotides as short as the dinucleotide.

Dilute aqueous solutions of poly U (*n*, about 200) and adenine tetranucleotide were adjusted to pH 7.2–7.4 with NaOH and mixed in varying proportions. At 10° in 10^{-3} *M* $MgCl_2$, the absorbancy of the mixtures at 259 mμ was less than that of the

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Abbreviations: Poly A, polyriboadenylic acid; poly U, polyribouridylic acid; poly I, polyriboinosinic acid; poly C, polyribocytidylic acid; DEAE cellulose, diethylaminoethylcellulose; $(pA)_4$, adenine terranucleotide with a 5′-phosphomonoester end group, also known as pApApApA.

components. The maximum decrease (about 35 %) occurred when the base ratio was 2 U to 1 A, or 0.66 mole fraction U. A change of similar size is found when poly A is mixed with poly U in this same molar ratio[5]. X-ray diffraction studies indicate the presence of a three-stranded helical complex in the latter polymer mixture[6]. A similar structure for the $(pA)_4$–poly U complex is probable and other evidence is consistent with the formation of a rather strong stoichiometric 2 U + 1 A complex with a high degree of order. Experiments carried out over a 30-fold range of concentration show the same minimum (at 0.66 mole fraction U) and the same degree of hypochromicity (about 35 %). Ultracentrifugal studies performed below 10° on $(pA)_4$–poly U mixtures at 0.66 mole fraction U showed that no $(pA)_4$ remained in the supernatant solution after centrifugation, whereas a pure $(pA)_4$ solution did not sediment under the same conditions.

The temperature at which disruption of the bonding between polynucleotides occurs has been used as a measure of the strength of such bonding. The absorbancy of a $(pA)_4$–poly U mixture (0.66 mole fraction U) was measured at 259 mμ as the temperature of the solution was increased (Fig. 1). The curve has the sigmoid shape typical of polynucleotide helix → coil transitions and shows no hysteresis. A similar curve relating optical rotation to temperature was found (Fig. 1), with the midpoint occurring at the same temperature. The specific rotation decreased from 1072° to 138° at 365 mμ and from 284° to 37° at 589 mμ as the temperature of the solution was raised from 20° to 51°. These changes are of the order of magnitude found for other polynucleotide complexes[7,8]. The exact value for the dissociation temperature (T_m), *i.e.*, the point of greatest slope of the curve, varies with the nucleotide concentration. Under the same conditions of salt concentration and pH, the T_m increased from 32.5° to 40.3° as the concentration of nucleotide phosphate was raised from 0.06 m*M* to 1.98 m*M*.

The stability of the adenine oligonucleotide–poly U complex increases markedly with oligonucleotide chain length. The T_m values for the $(pA)_n$ series of adenine oligonucleotides bonded to aliquots of the same lot of poly U have been determined

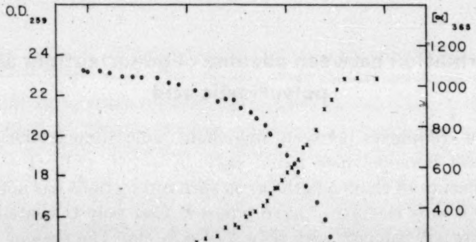

Fig. 1. Changes in absorbancy at 259 mμ (×) and optical rotation (±) with temperature in a solution of $(pA)_4$-poly U, 0.66 mole fraction U. The concentration of nucleotide (as phosphate) is 1.98 m*M*; the solvent is 1 *M* NaCl, 10^{-3} *M* $MgCl_2$, and 2 × 10^{-3} *M* potassium phosphate, pH 7.4.

in solutions of comparable salt and polymer concentrations. A plot of $1/n$ against T_m is linear (Fig. 2), which is what would be predicted on the basis of the Schellman model of cooperative order → disorder transitions[9]. However, the maximum slopes of the heating curves change much less rapidly with n ($\Delta f/\Delta T$ at T_m is 0.257 for n about 200, 0.229 for $n = 7$, and 0.172 for $n = 3$) than would be expected theoretically for a simple cooperative process[9] and than has been observed experimentally with polypeptides[10]. Therefore, in order to interpret in detail the shapes of the heating curves and their dependence upon polynucleotide concentration, it will be necessary to modify the Schellman model to include factors such as an association–dissociation equilibrium and the chain lengths of both components.

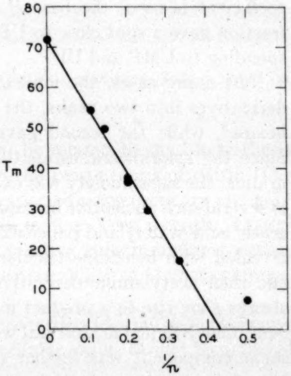

Fig. 2. Variation in dissociation temperature with reciprocal chain length of adenine oligonucleotide bonded to poly U at 0.66 mole fraction U. The solvent is the same as in Fig. 1; the nucleotide concentration is 0.06 mM (as organic phosphate). The value for the dinucleotide is based on a solution containing 0.50 mole fraction U, which gave the maximum hypochromicity observed for this oligonucleotide (—23 % at 2°).

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Uridine diphosphate derivatives in the tissues and hemolymph of insects

In the course of investigations of the acid-soluble phosphates of giant silkworm (*Saturniid*) pupae, our attention was attracted to a nucleotide fraction which occurs in rather high concentration. On the basis of its having an ultraviolet absorption maximum near 260 mμ and an apparent base: phosphate ratio, using the extinction coefficient of adenine, of approximately 1:3, this fraction, isolated from insect blood, was designated in an earlier publication[1] as ATP. This identification has proved to be incorrect. Analyses of representative samples are shown in Table I: the nucleotides have the spectral characteristics of uridine, and, when the appropriate extinction coefficient is used, the base:P ratio is 1:2. In paper electrophoresis (pH 3.5) the fraction gave a spot close to UDPGluc, and, after mild acid hydrolysis, spots corresponding to UMP and UDP.

In many cases, the ion-exchange chromatograms partially resolved the UDP derivatives into two peaks, the first and larger of which contained N-acetylaminohexose[3], while the second gave the anthrone reaction for unsubstituted hexose. Since the colorimetric estimates of Gluc and GlucNAc were always less than the uridine, the sugar moiety was examined further. The products of mild acid hydrolysis of several such nucleotide fractions were subjected to paper chromatography (butanol–acetic acid–water) and paper electrophoresis (sodium borate at pH 10[4]), spots being revealed with benzidine trichloroacetate. In the latter system Gluc, Gal, mannose and their acetylamino derivatives separate cleanly. The nucleotides of insect origin always gave rise to a product migrating with GalNAc, usually as the major component. GlucNAc, Gluc, and Gal were in some cases also present. Identity of the major sugar component was further confirmed by exposing a sample of the nucleotide from hemolymph to hydrolysing and deacetylating conditions (6 *N* HCl) followed by reaction with ninhydrin[5]. The principal sugar product migrated in borate electrophoresis identically with a lyxose standard prepared by treating a known sample of galactosamine in the same way. The product from glucosamine and from mannosamine is arabinose. We conclude that the principal component of the nucleotide fraction is UDPGalNAc.

The level of UDP sugar derivatives in *Cecropia* hemolymph changes characteristically with development of the insect. The larva has very little (about 0.01 μmole nucleotide/ml), the diapausing pupa 0.2–0.9 μmole/ml, and with the initiation of adult development this rises to about 1.5 μmoles/ml. These concentrations refer to the cell-free plasma, in which the predominant species found is UDPGalNAc. In the pupal fat body, the level both in diapause and early adult development is about 0.2 μmole/g, and includes more nearly equal proportions of the several sugars mentioned above. In the wing epidermis, concentrations are especially high, ranging from 0.8 μmole/g in diapause up to about 4 μmole/g in early adult development. This last value is possibly the highest concentration of UDP sugar derivatives recorded for any animal tissue; hen oviduct, by comparison, contains a total level of about 0.5 μmole/g[6].

Abbreviations: ATP, adenosine 5′-triphosphate; UMP and UDP, uridine 5′-monophosphate and -diphosphate; Gluc, glucose; Gal, galactose; GlucNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine.

TABLE I

CHARACTERIZATION OF UDP DERIVATIVES FROM INSECTS

Tissue extracts were prepared with cold 0.3 N $HClO_4$ and chromatographed on Dowex-1 formate[2]. The UDP derivatives were eluted with a gradient of $HCOONH_4$ in 1 N HCOOH.

Source	*λmax.*		*A_{280}/A_{260}*		*Moles/2.00 moles total P*		
	pH 1	*pH 13*	*pH 1*	*pH 13*	*Labile P*[*]	*Uridine*	*"GlucNAc"*[**]
Telea polyphemus							
pupal hemolymph	261	260	0.39	0.37	0.96	1.17	0.44
Platysamia cecropia							
pupal fat body	262	261	0.37	0.34	1.33	0.96	0.36
Known UDPGluc	261	260	0.39	0.33	0.98	0.99	—

[*] 1 N H_2SO_4, 100°, 15 min.

[**] Method of REISSIG, STROMINGER AND LELOIR[3] with GlucNAc standard. Values based on GalNAc standard would be 2.7 times higher.

The presence of UDPGluc in insect tissues may be related to the biosynthesis of trehalose and glycogen, that of UDPGlucNAc to the biosynthesis of chitin. The finding of the Gal and GalNAc nucleotides was less expected since there are virtually no reports of compounds of these sugars produced in insects. Very recently, LIPKE[7] has demonstrated insect hemolymph glycoproteins yielding galactosamine together with other sugars, and the present results suggest the desirability of further examination of insect tissues for mucopolysaccharides.

Also interesting is the selective occurrence of these nucleotides in the hemolymph plasma. By contrast, ATP, which is abundant in the cellular tissue, cannot be detected in the plasma.

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Stimulation of genetic transformation in *Bacillus subtilis* by biologically inactive DNA and polyphosphate

The biochemical mutants of *B. subtilis* Marburg strain may repair their nutritional defects on exposure to highly polymerized DNA, extracted from the normal strain[1]. This preliminary note will describe our results concerning the stimulatory effect of some substances on the phenomenon.

Throughout the experiments, a tryptophan-dependent and streptomycin-resistant mutant (160 s^r) of Marburg strain (courtesy of D. M. BONNER, Yale Univ.) was employed exclusively as the recipient cell of the DNA. The DNA was extracted from lysozyme protoplasts of nicotinic acid-dependent mutant cells with 10% NaCl solution in the presence of sodium citrate. After treatment with RNase, the crude extract was deproteinized by repeated applications of Sevag's method, and the DNA was finally precipitated with 2 vol. ethanol.

The method of DNA infection was as follows[1]: the spores of 160 s^r strain were inoculated into a glucose minimal medium containing 0.1 % of Difco yeast extract. After 5-h incubation at 37° with shaking, the cells were harvested and subcultured at a concentration of $5 \cdot 10^8$ cells/ml in the synthetic medium containing 0.01 % of yeast extract. DNA was added at a concentration of about 20 μg/ml to the subculture after one–half cell division occurred, as determined turbidometrically. The transformants were detected by spreading the DNase-treated aliquots of the cells and DNA mixture onto plates containing synthetic agar medium. This enzymic treatment immediately prevents further increase of transformants.

The transformants appear after a short lag period (5–15 min), and increase linearly with time (Fig. 1). The slope of the linear increase of transformants is proportional to the concentration of the DNA so long as it is below the saturation quantity (about 20 μg/ml). These findings are quite similar to the results obtained in the pneumococcal transformation experiments[2].

The lag period before the linear increase of transformants may be attributed to an adaptive formation of DNA-permeating sytem. To examine the possibility, the recipient cells were treated with the homologous DNA, which was extracted from cells identical with recipients, prior to the addition of transforming DNA. As is shown in Fig. 1, the pretreatment with homologous DNA preparation somewhat shortened the lag period, besides the stimulation of transformation. This suggested that a DNA-permeating system might be induced in recipient cells during the contact with DNA. Further study of the inducible formation of a permeating system was carried out using 8-azaguanine ($2 \cdot 10^{-4}$ M) or 2,4-dinitrophenol ($5 \cdot 10^{-4}$ M). These inhibitors did not generally influence the linear increase of transformants at concentrations inhibitory for the growth or the formation of amylase[3]. This fact was not reconcilable with the general conceptions of inducible enzymes.

The next approach to the analysis of the lag period and the effect of homologous DNA was suggested from two experimental results. First was the fact that the inducing effect of the homologous DNA preparation was insensitive to DNase treatment. This indicated the presence of some effective compounds other than highly polymerized DNA. The second was obtained from the experiments in which ^{32}P-labeled DNA preparations were used. The experiments demonstrated that *B. subtilis*

Abbreviations: DNA, deoxyribonucleic acid.

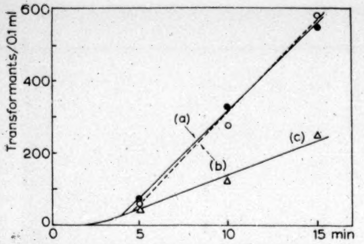

Fig. 1. About $8 \cdot 10^8$ cells/ml of tryptophan-dependent (160 s^r) *B. subtilis* were exposed at zero time to 15 μg/ml of transforming DNA with or without pretreatment. (a), 160 s^r-DNA which was digested with DNase (1 μg/ml) for 5 min at 37° was used; (b), 160 s^r cells were exposed to 15 μg/ml of 160 s^r-DNA for 15 min, followed by treatment with DNase and trypsin. After removal of enzymes by centrifugation, transforming DNA was added to the cell resuspension; (c), without pretreatment.

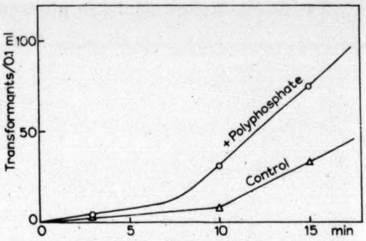

Fig. 2. Recipient cells were pretreated with 18 μg/ml of sodium polyphosphate for 15 min. Transforming DNA was added without removal of polyphosphate.

adsorbed an insoluble ^{32}P-compound immediately after mixing the cells with ^{32}P-labeled DNA[4]. This phosphorus compound was thought to be polyphosphate, since a large amount of polyphosphate was shown to accumulate in *B. subtilis* N[5], with the possibility that it contaminated our DNA preparations. The polyphosphate which was synthesized from NaH_2PO_4 (chain-length approximately 30 phosphates, as determined titrimetrically) was quite effective in transformations even at a low concentration (18 μg/ml) (Fig. 2). Although the effect of polyphosphate resembled that of homologous DNA preparations we cannot at present simply attribute the effect of homologous DNA preparations to contamination by polyphosphate. The phenomenon may be related to the reactivation of dialysed serum factor by pyrophosphate observed in pneumococcal transformation[6]. Further research on this problem is now under way.

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Étude de l'hémoglobine plasmatique de quelques Annélides au microscope électronique

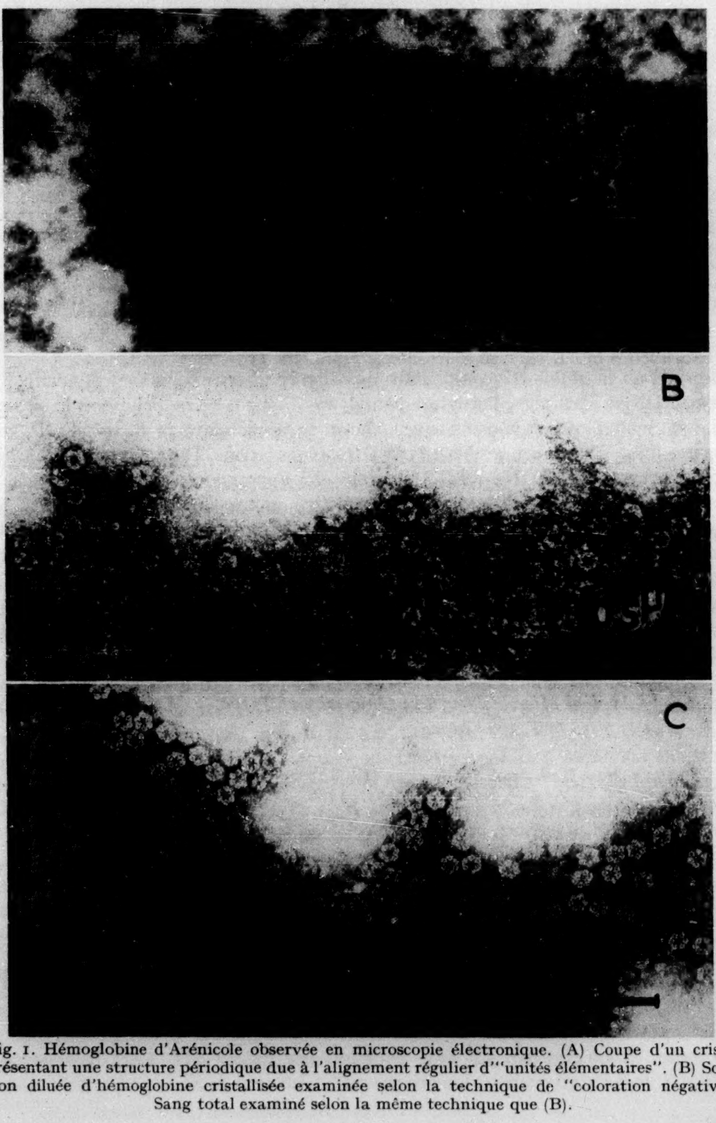

Fig. 1. Hémoglobine d'Arénicole observée en microscopie électronique. (A) Coupe d'un cristal présentant une structure périodique due à l'alignement régulier d'"unités élémentaires". (B) Solution diluée d'hémoglobine cristallisée examinée selon la technique de "coloration négative". Sang total examiné selon la même technique que (B).

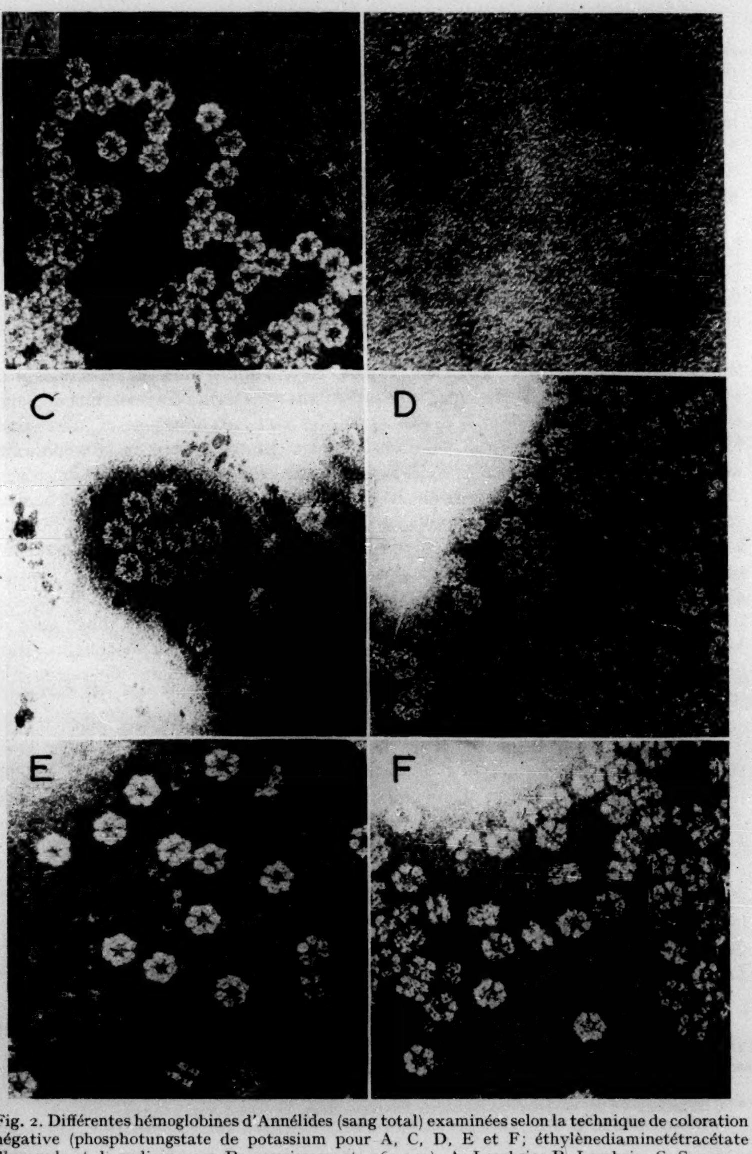

Fig. 2. Différentes hémoglobines d'Annélides (sang total) examinées selon la technique de coloration négative (phosphotungstate de potassium pour A, C, D, E et F; éthylènediaminetétracétate d'uranyle et de sodium pour B; grossissement: 260,000). A. Lombric; B. Lombric; C. Sangsue; D. Arénicole; E. *Lanice*; F. *Nereis*.

Le sang de certains Vers renferme, à l'état dissous, et comme constituant protéique de beaucoup le plus important, une hémoglobine dont le poids moléculaire est bien plus élevé que celui des pigments de même type localisés dans les hématies d'autres Annélides, de Mollusques et de tous les Vertébrés[1,2]. L'ultracentrifugation de ces hémoglobines extracellulaires a conduit à leur attribuer un poids moléculaire de 2,750,000, alors que celui de toutes les hémoglobines endoglobulaires est égal à 68,000 ou, exceptionnellement, à 34,000 et 17,000. Ces hémoglobines de haut poids moléculaire nous ont paru mériter d'être étudiées par microscopie électronique.

De l'hémoglobine cristallisée préparée à partir du sang d'un Annélide Polychète (*Arenicola marina* L.) a fait l'objet d'une première série d'examens[3]. On a pu constater l'existence d'une structure périodique sur des coupes de cristaux fixés par le tétroxyde d'osmium (Fig. 1, A). L'étude de solutions très diluées obtenues à partir d'hémoglobine cristallisée, a été faite par la méthode de "coloration négative"[4]. Ces dilutions sont mélangées à une solution de phosphotungstate de potassium, puis pulvérisées sur un support de formwar. Les micrographies électroniques montrent des images de molécules isolées (Fig. 1, B). Lesquelles se présentent sous forme d'association de particules dont le diamètre est de 70 Å environ, groupées par six en hexagones réguliers, dont le diamètre est de 230 Å environ. On observe également sur le fond de la préparation un certain nombre de particules isolées ou groupées en amas de moins de six unités.

Ces différents aspects se retrouvent dans des préparations de sang fraîchement prélevé d'Arénicole, dilué sans avoir subi aucun traitement. Les résultats obtenus sont reproduits sur la Fig. 1, C. Le pigment ne paraît constitué, presque uniquement, que par des groupes hexagonaux de six particules. Vus de face, ceux-ci offrent l'aspect de disques; vus par la tranche, ils se présentent comme de petits bâtonnets associés par deux. Ils se disposent fréquemment à la manière de "rouleaux" d'hématies de Vertébrés. Des images analogues ont été obtenues en substituant au phosphotungstate de potassium de l'étylènediaminetétracétate d'uranyle et de sodium[5]. On retrouve la même structure après ombrage au platine-carbone.

Des observations identiques ont été faites sur les hémoglobines plasmatiques d'autres Annélides Polychètes marins (*Nereis diversicolor, Lanice conchilega*) d'un Oligochète terrestre, le Lombric (*Lumbricus* sp.), et d'un Hirudiné, la Sangsue (*Hirudo medicinalis*) (Fig. 2). Leur taille semble identique à 10% près, la marge d'erreurs dues à l'appareillage et à la technique ne permettant pas pour le moment d'attribuer de signification à des différences de cet ordre.

Ces aspects en rosettes de six éléments semblent donc communs au pigment respiratoire de plusieurs espèces de Vers. Des recherches en cours montreront s'ils se retrouvent ou non dans les chlorucruorines et dans d'autres hémoglobines extraglobulaires.

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PREPARATION AND PROPERTIES OF THE IRON-PROTOPORPHYRIN CHELATING ENZYME

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SUMMARY

Further studies are reported on the iron-protoporphyrin chelating enzyme. The enzyme was prepared in soluble form from rat-liver mitochondria and was assayed spectrophotometrically. These procedures and numerous properties of IPCE are described. The enzyme is quite stable, has maximum activity at pH 8.2 and the only established cofactor requirement is a reducing agent such as glutathione. It exhibits numerous properties of an enzyme requiring a sulfhydryl group for activity.

INTRODUCTION

The last reaction in the biosynthesis of protoheme involves the chelation of a ferrous ion by protoporphyrin. This is an enzymically catalyzed reaction which may be represented as follows:

$$\text{Protoporphyrin} + \text{Fe}^{++} \longrightarrow \text{Heme} + 2\text{H}^{+}$$

Although the release of two protons remains to be experimentally established, this would be predicted on a basis of the reaction stoichiometry. Previous reports from this laboratory have described some of the properties of the iron-incorporating enzyme prepared from rat-liver mitochondria[1,2]. Its preparation in soluble form and a spectrophotometric assay for determining activity have been reported briefly[2]. MINAKAMI *et al.*[3] have also studied the incorporation of iron into heme by liver preparations. KRUEGER *et al.*[4], KAGAWA *et al.*[5] and SCHWARTZ *et al.*[6,7] have prepared the enzyme in soluble form from avian erythrocytes and observed many of its properties. GOLDBERG[8] has investigated primarily the roles of glutathione, ascorbic acid and ergothioneine in the reaction. The present communication describes in more detail preparation of the enzyme from rat liver, its assay and additional observations bearing on its properties and mode of action. It is referred to here as the iron-protoporphyrin chelating enzyme.

MATERIALS AND METHODS

Adult Sprague-Dawley or Wistar rats of either sex were decapitated, bled, and the livers removed and chilled immediately in ice. The livers were homogenized in nine

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The following abbreviations are used: iron-protoporphyrin chelating enzyme (IPCE), protoporphyrin (PROTO), tris(hydroxymethyl)aminomethane (Tris), diphosphopyridine nucleotide (DPN), 2,3-dimercapto-1-propanol (BAL), reduced and oxidized glutathione respectively (GSH, GSSG), allylisopropylacetamide (AIA), *p*-chloromercuribenzoate (PCMB).

volumes of 0.25 M sucrose with a Waring blendor. Mitochondria were separated by fractional centrifugation and suspended in four volumes of 0.10 M $KHCO_3$. All operations were carried out at 5°. Storing the mitochondria at — 20° is permissible and usually improved the later extraction of the enzyme. The enzyme was obtained in soluble form by adding to the mitochondrial suspension Tween 20 to a concentration of 10 mg/ml. The mixture was allowed to stand in an ice bath for 30 min with occasional stirring. It was then centrifuged at 36,000 × g for 30 min at 0°. A clear reddish supernate was obtained. It was stored at — 20° until used. Some purification can be accomplished by washing the mitochondria prior to liberation of the enzyme.

PROTO was prepared by the method of GRINSTEIN[9]. It was further purified by dissolving in 0.15 M $KHCO_3$, centrifuging out insoluble material, and adjusting to pH 4–5 with glacial acetic acid. After its precipitation in the cold, the PROTO was centrifuged out, dissolved in 3 N HCl and insoluble material again centrifuged out. The PROTO was precipitated with sodium acetate, washed with water and redissolved in 0.15 M $KHCO_3$ to make a suitable concentration. This stock solution was stored at — 20°. DPN was purchased from the Sigma Chemical Company, St. Louis, Missouri. AIA was generously supplied by Hoffmann-LaRoche, Inc., Nutley, New Jersey and Tween-20 by the Atlas Powder Company, Wilmington, Delaware.

IPCE was assayed spectrophotometrically by incubating the following: enzyme (0.4 ml), PROTO (0.02 μmole), $FeSO_4$ (0.02 μmole), Tris (100 μmoles) and reducing agent (40 μmoles) in a total volume of 1.0 ml. These quantities may be varied within limits to suit the experiment. Incubation was carried out under nitrogen at 38° for periods up to 2 h. Following incubation 9 volumes of 1.5 N HCl were added and the precipitated protein centrifuged out. Spectral absorption of the supernate at 407 mμ was measured. A sample without enzyme was included in each assay and the amount of PROTO utilized in the enzymic reaction was calculated from the decrease in absorption (ΔA_{407}). An assay using ^{59}Fe was described previously[1] and has been used by others investigating this reaction[3–8].

RESULTS

Assay

The spectrophotometric assay was based upon the disappearance of PROTO. In order to show that PROTO actually chelated iron and was converted to heme rather than entering into a side reaction, simultaneous assays were carried out in a variety of instances measuring both ΔA_{407} and ^{59}Fe uptake. As reported previously[2] ΔA_{407} parallels the incorporation of ^{59}Fe, thus showing that the PROTO disappears at the same rate as ^{59}Fe appears in heme. An example is illustrated by the data in Fig. 3. Although heme and PROTO have similar absorption characteristics, heme does not interfere in the spectrophotometric assay described because of its insolubility in HCl and precipitation with the protein. In the absence of enzyme there was no measurable decrease in PROTO under assay conditions. In the absence of added PROTO the enzyme itself had an $A_{407} < 0.010$ and therefore offered no interference in the assay. Occasionally samples containing very small amounts of protein or much lipid did not precipitate well but rather produced a turbid solution. It was found that such difficulties could be circumvented by simply making appropriate dilutions of the supernates (about 30 fold) and measuring the unreacted PROTO fluorometrically which gave results paralleling the spectrophotometric measurements. Calculating from a molar extinction coefficient of $1.93 \cdot 10^5$ the PROTO data can be expressed as mμmoles utilized as well as ΔA_{407}.

Soluble enzyme

IPCE as prepared contained 6–8 mg of protein/ml. Even at this relatively low protein concentration the enzyme showed no loss of activity after storage for several weeks at — 20°. This is much more stable than the enzyme derived from avian erythrocytes[5,6]. Nor did repeated freezing and thawing decrease its activity, although some denatured protein occasionally precipitated. IPCE was stable to lyophilization, prolonged blendorizing and at least 18 h dialysis against Tris buffer (pH 8.2). The enzyme was firmly bound in the mitochondria and both the Tween 20 and a salt in combination were necessary for its extraction. Other Tweens tested were less effective and desoxycholate interfered with the spectrophotometric assay. IPCE lost only about 10 % of its activity when heated to 56° for 30 min, but was rapidly inactivated at 60° (Table I). Centrifuging IPCE at 140,000 × *g* for 2 h resulted in 38 % of the enzyme sedimenting into the residue (Table II). Thus, some of it may have been suspended as a large micelle in the Tween 20.

After incubating IPCE and ^{59}Fe in the absence of PROTO, no radioactive hemin could be isolated showing a complete absence of endogenous PROTO or precursors convertable to PROTO. δ-aminolevulinic acid was not converted to heme in this system, which is an important difference from the avian erythrocyte preparation of KAGAWA *et al.*[5]. When no iron was added, the better preparations had about one-third of maximum activity based on PROTO utilization (Fig. 1).

TABLE I

HEAT INACTIVATION

Conditions: 24 mμmoles PROTO, 24 mμmoles Fe^{++}, 40 μmoles GSH, 100 μmoles buffer, 0.50 ml enzyme, H_2O to 1.0 ml, incubated 2 h.

*Temperature**	*mμmoles PROTO utilized*
Unheated	12.1
56°	11.2
60°	4.8
64°	2.9
100°	0

* Heated 10 min.

TABLE II

SOLUBILITY OF ENZYME

Conditions: 27 mμmoles PROTO, 36 mμmoles Fe^{++}, 40 mμmoles ascorbic acid, 200 mμmoles buffer, 0.4 ml enzyme, H_2O to 1.0 ml, incubated 1 h.

Preparation	*mμmoles PROTO utilized*	*% activity*
36,000 × *g*, 30 min, supernate	15.2	100
140,000 × *g*, 120 min, supernate	8.5	56
140,000 × *g*, 120 min, residue	5.8	38

pH response

The pH optimum of the enzyme in particulate form was reported previously[1] as being 7.4. However, a re-evaluation with the soluble preparation described here showed the greatest IPCE activity at about pH 8.2, although the shape of the pH curve was essentially the same in both cases. Phosphate, barbital and Tris buffers behave similarly, the latter in acetate form having been chosen for all succeeding work.

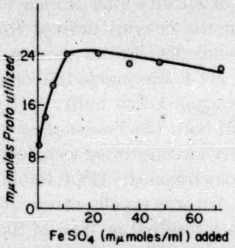

Fig. 1. Iron concentration curve. 30 mμmoles PROTO, 200 μmoles buffer, 40 μmoles ascorbic acid, 0.4 ml enzyme, H_2O to 1.0 ml, incubated 1 h.

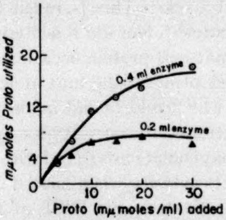

Fig. 2. Protoporphyrin concentration curve. 30 μmoles Fe^{++}, 100 μmoles buffer, 40 μmoles ascorbic acid, 0.4 ml enzyme, H_2O to 1.0 ml, incubated 1 h.

Substrates and cofactors

Maximum activity and enzyme saturation was obtained with an iron concentration of $2 \cdot 10^{-5}$ *M* (Fig. 1). The enzyme contains some residual iron which is evidently available for heme formation. A PROTO concentration of about $3 \cdot 10^{-5}$ *M* is optimum (Fig. 2). The value depended of course on enzyme concentration as shown. More than 80 % reaction based on PROTO utilization has been observed.

In addition to cofactor studies reported previously[1,2] compounds further tested and shown to be nonstimulatory include citrate, adenosine, inosine, thymidine and vitamin B_{12}. The stimulation due to histidine reported for the particulate preparation[1] was not seen in the soluble preparation. The addition of heat inactivated enzyme was without effect.

It was reported previously that DPN stimulated iron incorporation several fold[1]. This same effect was seen in the soluble IPCE preparation where DPN was active in 1/100th the concentration of ascorbic acid (Table III). It should be noted, however, that DPN stimulation was not additive with that due to ascorbic acid or GSH.

TABLE III

EFFECTS OF DPN AND ASCORBIC ACID

Conditions: 24 mμmoles PROTO, 24 mμmoles Fe^{++}, 100 μmoles buffer, 0.5 ml enzyme, H_2O to 1.0 ml, incubated 2 h.

Ascorbic acid μmoles	*DPN μmoles*	*PROTO utilized mμmoles*
		2.8
40		18.0
	0.4	12.6
40	0.4	18.5

Only a 10 % increase in activity could be obtained with $2 \cdot 10^{-4}$ *M* ergothioneine in the presence of excess ascorbic acid or GSH. Thus any action which ergothioneine exhibited was due to an effect other than the reducing action of its sulfhydryl group; but its effect is small and remains to be established as a participant in the reaction. Allylisopropylacetamide (AIA), a drug used to produce experimentally the liver disease acute intermittent porphyria, also stimulated the incorporation of iron in partially purified IPCE as prepared here (Fig. 3). This effect was not seen in liver homogenate.

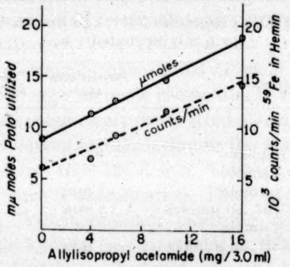

Fig. 3. Effect of allylisopropylacetamide. 72 mμmoles PROTO, 72 mμmoles Fe^{++} (including ^{59}Fe), 300 μmoles buffer, 120 μmoles ascorbic acid, 1.2 ml enzyme, H_2O to 3.0 ml, incubated 1 h. Following incubation 1.0 ml used for spectrophotometric assay and 2.0 ml used for isotopic assay.

Inhibitors

IPCE was inhibited more than 75 % by metal-binding agents such as $2.5 \cdot 10^{-2}$ *M* cyanide, $8 \cdot 10^{-3}$ *M* BAL and ethylenediaminetetracetic acid (EDTA) at $< 5 \cdot 10^{-4}$ *M*. Bilirubin inhibited in part at least by competition with PROTO[10].

IPCE was inactivated by oxygen and GSSG. Conversely it was activated by incubation under nitrogen and, as reported previously[1], by GSH, homocysteine (or cysteine) and ascorbic acid. Preincubation of IPCE with a reducing agent in ice was necessary for maximum activity during short reaction periods. Ascorbic acid was about twice as stimulatory as GSH on a molar basis and effects of the two were additive. IPCE was inhibited by iodoacetate, iodacetamide and PCMB (Table IV). When GSH

TABLE IV

SULFHYDRYL GROUP INHIBITORS

Conditions: 24 mμmoles PROTO, 24 mμmoles Fe^{++}, 100 μmoles buffer, 0.4 ml enzyme, H_2O to 1.0 ml, incubated 2 h.

Inhibitor	*μmoles*	*mμmoles PROTO utilized*
		6.3
PCMB	0.10	4.5
	0.20	2.0
		6.5
Iodoacetamide	0.10	3.8
	0.20	2.8
		4.0
Iodoacetic acid	0.10	3.3
	0.20	3.1

was added with PCMB, there was no inhibition, but if the enzyme was first inactivated with PCMB, then GSH could not reverse the inhibition (Table V). The amount of inhibition by PCMB was decreased in the presence of greater PROTO concentrations (Table VI) but was unaffected by iron concentration. BAL, which is commonly used as a sulfhydryl group activator, actually was a potent inhibitor for IPCE as noted above.

TABLE V

EFFECTS OF PCMB AND GSH IN COMBINATION

Conditions: 30 mμmoles PROTO, 24 mμmoles Fe^{++}, 140 μmoles buffer, 0.3 ml enzyme, H_2O to 1.0 ml, incubated 1 h.

Addenda	*Preincubation at 0°*	*mμmoles PROTO utilized*
		5.3
GSH, 40 μmoles		18.9
PCMB, 0.3 μmole		3.1
PCMB, 0.3 μmole	15 min	
then GSH, 40 μmoles	15 min	3.6
PCMB, 0.3 μmole		
plus GSH, 40 μmoles	30 min	20.1

TABLE VI

EFFECT OF PROTO CONCENTRATION ON PCMB INHIBITION

Conditions: 30 mμmoles Fe^{++}, 100 μmoles buffer, 0.3 ml enzyme, 0.3 μmole PCMB where indicated, H_2O to 1.0 ml, incubated 1 h.

PROTO added mμmoles	*PROTO utilized*		*Per cent inhibition*
	— PCMB mμmoles	*+ PCMB mμmoles*	
17.4	5.3	1.2	77
36.0	7.2	2.8	61
52.3	8.8	3.7	58

DISCUSSION

The data obtained in this laboratory and elsewhere[1-8] establish the existence in several tissues of a soluble enzyme which catalyzes the chelation of iron by protoporphyrin to form heme. Where comparable data are available, the liver enzyme and the avian erythrocyte enzyme have a number of properties in common. With the liver enzyme, however, no evidence for more than one component has yet been obtained while the chicken erythrocyte preparation has been divided into two heat labile, nondialyzable fractions[7], but one of these fractions might conceivably stimulate the reaction without participating in it as an enzyme.

Although no specific conclusions can be made concerning the mechanism of this chelation reaction, certain observations obtained to date are pertinent. With the exception of a reducing agent for sulfhydryl activation, no specific cofactors have yet been established as being essential for IPCE activity. While ascorbic acid, GSH and similar compounds are presumed to stimulate by activating sulfhydryl groups, the possibility is not excluded that one or more of them may perform an additional function such as keeping the iron reduced as suggested by GOLDBERG[8]. Although a great variety of other compounds have been tested, significant results have been noted only for the pyridine nucleotides[1]. At optimum concentration DPN produces only about 70 % of the stimulation due to GSH or ascorbic acid, and either of the reducing agents can obscure the effect of DPN. The role of DPN in this reaction is not clear, but it is entirely possible that DPN is affecting a secondary process which in turn activates IPCE. Further purification of IPCE may help to resolve this problem.

Ergothioneine has been reported to stimulate the incorporation of iron into heme by avian erythrocytes[8]. Although liver IPCE is also stimulated slightly by this compound, a variety of experiments have failed to yield conclusive evidence that ergothioneine is a cofactor. If ergothioneine is essential in the reaction, it is probably an enzyme component which is not easily dissociated. Certainly some very reasonable hypotheses can be made involving ergothioneine in the reaction.

There is little question but that IPCE is a "sulfhydryl" enzyme since it meets several of the criteria for such a classification. Thus, it is inhibited by alkylating reagents (iodoacetic acid, iodoacetamide), oxidizing reagents (oxygen, oxidized glutathione) and mercaptide-forming reagents (PCMB). While the role of a sulfhydryl group remains to be established, it may be involved with binding PROTO to an active site since increasing concentrations of PROTO do offer some protection against attack by PCMB, whereas, iron has no effect on this inhibitor.

The action of AIA in stimulating IPCE cannot be explained at this time. The finding is in agreement with the observation that porphyria-producing drugs not only increase porphyrin synthesis[11] but also stimulate iron uptake by the liver and its incorporation into heme[12]. Further study of these drugs seems indicated since certain other compounds related to AIA have a similar effect on IPCE.

ACKNOWLEDGEMENTS

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KONTRAKTIONSZYKLUS UND INTERAKTION ZWISCHEN AKTIN UND L-MYOSIN UNTER DER WIRKUNG SPEZIFISCHER INTERAKTIONS-INHIBITOREN

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SUMMARY

Contraction cycle and inhibitors of actin–L-myosin interaction

1. The term interaction inhibitor describes such substances that never affect L-myosin induced ATP splitting but that, when in the presence of ATP, do inhibit the enzymic interaction between actin and L-myosin, *i.e.*, they convert the Mg-activated actomyosin ATPase reversibly into L-myosin ATPase.

2. Examples of interaction inhibitors are the relaxing factor, discovered by MARSH and BENDALL, some polycations, polysulfonates, EDTA, and SCN^- in low concentrations.

3. It is shown for polyethensulfonate, EDTA, Fuadin, and SCN^- and confirmed for the relaxing factor that in the presence of ATP these substances inhibit reversibly the association of actin and L-myosin as well as their chemical interaction.

4. For the fibrils in the water–glycerol extracted fibers this is demonstrated by measuring the resistance to stretching.

5. For gels from natural actomyosin the same conclusion can be drawn, because the actin-component can be almost completely extracted from these gels by an ATP-containing polyethensulfonate solution.

6. Contracted water–glycerol extracted fibers relax as soon as the actin and L-myosin filaments dissociate through the action of ATP and an interaction inhibitor.

7. The dissociation of actin and L-myosin disappears if either the concentration of ATP or that of the interaction inhibitor is sufficiently lowered.

8. In both cases contraction and ATP splitting by Mg-activated actomyosin ATPase occurs anew along with the reappearing association.

9. In the presence of ATP and an interaction inhibitor relaxation even occurs if the Mg-activated actomyosin ATPase is converted into the highly effective Ca-activated L-myosin ATPase instead of into the slightly effective Mg-activated L-myosin ATPase.

Hence contraction is only produced when the mechanism of ATP splitting is based on the interaction between actin and L-myosin. Contraction is independent of ATP-splitting by L-myosin ATPase.

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EINLEITUNG

Gifte, die an den wirksamen Gruppen des L-Myosin angreifen, wie die SH-Reagenzien oder die Agentien, die die Aminogruppen des L-Myosin substituieren, hemmen sowohl die ATP-Spaltung durch L-Myosinsole wie L-Myosingele und hemmen ebenso die ATP-Spaltung durch Aktomyosinsole und Aktomyosingele. Diese Hemmung ist davon unabhängig, ob die Aktivierung durch Ca oder Mg erfolgt. Die Hemmung ist vollständig, falls die Konzentration der Inhibitoren genügend hoch ist. Wir nennen solche Gifte Myosin-ATPase-Gifte. Diesen Giften stehen andere Inhibitoren gegenüber, die die ATP-Spaltung durch L-Myosin nicht beeinflussen, dagegen die hohe Geschwindigkeit der Mg-aktivierten ATP-Spaltung durch Aktomyosingel auf die kleine Geschwindigkeit der ATP-Spaltung durch L-Myosin reversibel herabsetzen. Diese Art der Inhibitoren ist deswegen besonders interessant, weil der von Marsh[1,2] entdeckte physiologische Erschlaffungsfaktor zu ihnen gehört[3,4]. An chemisch bekannten Inhibitoren gehört zu dieser Gruppe das Polylysin[5], das Protamin, Heparin und Polyäthensulfonat[6], das Fuadin[7,*] und einige andere Interaktions-Inhibitoren, (EDTA und SCN^-) die in dieser Arbeit zusätzlich analysiert werden.

Es wird in dieser Arbeit gezeigt, dass alle Interaktions-Inhibitoren die dissoziierende Wirkung des ATP so sehr steigern, dass selbst kleine ATP-Konzentrationen die Affinität und damit die Interaktion zwischen Aktin und L-Myosin auch in Fibrillen und Aktomyosingelen vollständig aufheben. Es wird ferner gezeigt, dass die in dieser Arbeit untersuchten Interaktions-Inhibitoren ausschliesslich durch ihre Fähigkeit erschlaffend wirken, die ATP-induzierte Dissoziation des Aktomyosin zu aktivieren.

I

Hanson und Huxley[8] haben zuerst gezeigt, dass die Dehnung der Muskelfasern bei niedriger Ionenstärke auf einem Abgleiten der Aktinfilamente von den L-Myosinfilamenten beruht. Infolgedessen ist die Dehnung um so grösser und der Dehnungswiderstand um so kleiner, je geringer die Kohäsionskräfte zwischen Aktinfilamenten und L-Myosinfilamenten sind. Der Dehnungswiderstand wird Null, sobald diese Kohäsionskräfte Null sind, oder mit anderen Worten, sobald Aktin und L-Myosin vollständig dissoziert sind. Daher ist die Grösse des Dehnungswiderstandes ein Mass für den Grad der Dissoziation von Aktin und Myosin in der Muskelfibrille. Doch muss der Dehnungswiderstand bei einer Faserlänge gemessen werden ($\leqq$ Standardlänge), bei der die parallel geschalteten elastischen Elemente Hills[9] noch nicht gedehnt werden.

Unter diesen Bedingungen ist der Dehnungswiderstand der Wasser–Glyzerin-extrahierten Fasern immer dann sehr hoch ($7 \rightarrow > 10\ \mathrm{kg/cm^2} \times L \times \Delta L^{-1}$), wenn ATP oder andere Polyphosphate abwesend sind[10]. Dieser Dehnungswiderstand wird kleiner, sobald ATP anwesend ist[10]. Der Dehnungswiderstand wird Null in Gegenwart von $3 \cdot 10^{-3}\ M$ Mg, wenn die Konzentration des zugesetzten ATP $\geqq 10^{-2}\ M$ ist (Tabelle I). Gleichzeitig sinkt die Geschwindigkeit der Mg-aktivierten ATP-Spaltung vom Wert der Aktomyosin-ATPase auf den Wert der L-Myosin-ATPase (Fig. 1, Vergl. auch Perry und Grey[11], Fig. 5a). Aus beiden Beobachtungen ergibt sich

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[*] Eine Komplexverbindung von Antimon (III) mit 2 Molekülen Brenzkatechindisulfosäure.

TABELLE I

DEHNUNGSWIDERSTAND VON EXTRAHIERTEN EINZELFASERN IN ANWESENHEIT VON INTERAKTIONS-INHIBITOREN

Der Dehnungswiderstand wird nach einer Dehnung um 4–10% der Ausgangslänge bestimmt. Für die Messung des Dehnungswiderstandes in Anwesenheit von ATP werden Inhibitor und ATP gleichzeitig hinzugefügt. Die Messung ohne ATP erfolgt mit einer frischen Wasser–Glycerin-extrahierten Faser in einem neuen Bad, das nur den Inhibitor enthält. Die Ablesung erfolgt in beiden Fällen nach 10–15 Min. Diese Wartezeit ist notwendig, weil in Anwesenheit von ATP immer eine kurze Anfangskontraktion auftritt. Diese Anfangskontraktion tritt selbst dann ein, wenn der Inhibitor in Anwesenheit von $3\cdot10^{-3}$ *M* ATP die Kontraktion vollständig hemmt. (Erklärung siehe Abschnitt II). Die angegebenen Dehnungswiderstände sind nur bis auf 100 g/cm² × L × ΔL^{-1} gesichert, da kleinere Dehnungswiderstände unter die Ablesungsgenauigkeit des Lichtzeigers (1 mm Skala) fallen. (pH, 6.9; 0.05 *M* KCl; $3\cdot10^{-3}$ *M* $MgCl_2$; Temperatur, 25°).

Interaktions-Inhibitoren	*Ausgangs-Länge ($L/L_0 \times 100$)*	*Statischer Dehnungswiderstand ($g \times cm^{-2} \times L \times \Delta L^{-1}$)*		*Zahl der Versuche*
		Mit ATP	*Ohne ATP*	
ATP: $1\cdot10^{-2}$–$2\cdot10^{-2}$ *M*	50	~ 0	5000–10,000	10
		mit $3\cdot10^{-3}$ M ATP		
Erschlaffungsfaktor: 1–3 g/l	33–50	~ 0	> 10,000	7
Polyäthensulfonat: $5\cdot10^{-6}$–$1.5\cdot10^{-4}$ *M*	50	~ 0	4,500–7,000	19
EDTA: $3\cdot10^{-4}$ *M*	100	300		1
$1\cdot10^{-3}$–$1\cdot10^{-2}$ *M*	100	~ 0	> 10,000	4
KSCN: 0.003 *M*	50	2,000–2,500	5,000	4
0.01 *M*	100	250– 500	8,000	3
0.03 *M*	100	~ 0	> 10,000	3
0.1 *M*	100	~ 0	1,600	4

übereinstimmend, dass die Substrathemmung der ATP-Spaltung auf einer Dissoziation des Aktomyosins beruht.

Die volle Dissoziation des Aktomyosins wird schon von viel kleineren Konzentrationen von ATP als 10^{-2} *M* hervorgerufen, sobald ausser dem ATP ein Interaktions-Inhibitor anwesend ist (Tabelle I). Aber auch mit dem wirksamsten Interaktions-Inhibitor nimmt der Dehnungswiderstand zu, sobald die ATP-Konzentration sehr klein wird (Fig. 2). Wenn die ATP Konzentration ≙ Null ist, erreicht der Dehnungswiderstand selbst in Gegenwart der Inhibitoren den vollen Wert der Starre (ähnlich 10 kg/cm² × L × ΔL^{-1}), (Tabelle I, Fig. 2).

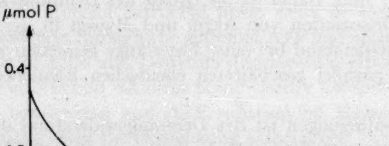

Fig. 1. Die Verwandlung der hochaktiven Aktomyosin-ATPase in die wenig aktive L-Myosin-ATPase durch ATP in überoptimalen Konzentrationen in Gegenwart von $3\cdot10^{-3}$ *M* Mg. Ordinate: Spaltungsgeschwindigkeit in μmol P/mg Fibrilleneiweiss × min. Abszisse: ATP-Konzentration (0.05 *M* KCl; 0.01 *M* Tris–Maleinat-Puffer pH 6.9; Temperatur, 25°).

Die Unbeeinflussbarkeit des Dehnungswiderstandes durch Inhibitoren ohne ATP ist in unseren Versuchen festgestellt bis zu einer Konzentration der Interaktions-Inhibitoren von $3 \cdot 10^{-2}$ *N** (Tabelle I). Die Normalität von $3 \cdot 10^{-2}$ *N* errechnet sich aus den Höchstwerten der Molarität in Tabelle I, in der für Polyäthensulfonat eine Wertigkeit von 205 eingesetzt ist.

Die Beobachtungen zeigen, dass die Dissoziation der Aktin- und L-Myosinfilamente (a) vom ATP bewirkt wird, und (b) dass diese dissoziierende Wirkung des ATP von den Interaktions-Inhibitoren gefördert wird.

Infolgedessen kann die Wirksamkeit eines Interaktions-Inhibitor auf zwei Arten beurteilt werden. (a) Durch Messung der niedrigsten ATP-Konzentration, die in Gegenwart einer konstanten Konzentration des Inhibitors den Dehnungswiderstand auf Null herabsetzt. (b) Durch die niedrigste Konzentration des Inhibitors, die in Anwesenheit einer konstanten ATP-Konzentration den Dehnungswiderstand auf Null herabsetzt. Ergebnisse der ersten Methode können aus Fig. 2, Ergebnisse der zweiten Methode aus Tabelle I entnommen werden. Auf Grund dieser Ergebnisse ordnet sich die Wirksamkeit der verschiedenen Inhibitoren in der folgenden Reihenfolge**, falls deren Konzentrationen als molare Konzentrationen gemessen werden: Polyäthensulfonat > EDTA > Fuadin > KSCN

Für einen bestimmten Interaktions-Inhibitor wächst die Wirkung auf den ATP induzierten Dissoziations-Zustand mit der Konzentration (Tabelle I, Fig. 2).

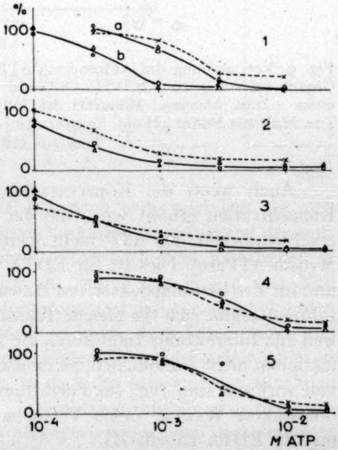

Fig. 2. Die Abhängigkeit der isometrischen Spannung, des Dehungswiderstandes und der ATP-Spaltung von der ATP Konzentration in Anwesenheit von konstanten Konzentrationen verschiedener Interaktions-Inhibitoren. Ordinate: × --- ×, Spaltungsgeschwindigkeit; △—△, Dehnungswiderstand; O—O, Isometrische Spannung in % des unbeeinflussten Wertes (Erklärung siehe im Text). (1) Erschlaffungsfaktor (a) 1.0 mg/ml; (b) 2.0 mg/ml. (2) $2 \cdot 10^{-5}$ *M* Polyäthensulfonat. (3) $1 \cdot 10^{-3}$ *M* EDTA. (4) $1 \cdot 10^{-3}$ *M* Fuadin. (5) $4 \cdot 10^{-3}$ *M* KSCN. Abszisse: ATP–Konzentration. ($3 \cdot 10^{-3}$ *M* $MgCl_2$; 0.05 *M* KCl; 0.01 *M* Tris–Maleinat-Puffer pH 6.9; Temperatur, 25°; Einzelfasern: 40–50 μ Durchmesser; 40 % Anfangslänge).

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* In einer noch höheren Konzentration wurde nur KSCN untersucht. 0.1 *M* KSCN vermindert auch ohne ATP den Dehnungswiderstand deutlich (Tabelle I). 0.3 *M* KSCN vernichtet den Dehnungswiderstand vollständig. KSCN ist also ein Interaktions-Inhibitor ohne Nebenwirkungen nur in Konzentrationen kleiner als 0.1 *M*.

** Diese Reihenfolge ergibt sich für alle Inhibitoren ausser Fuadin aus Tabelle I. Dass Fuadin etwas wirksamer ist als KSCN folgt daraus, dass die Hemmung durch $1 \cdot 10^{-3}$ *M* Fuadin für alle ATP-Konzentrationen ebenso gross ist wie die Hemmung durch $4 \cdot 10^{-3}$ *M* KSCN (Fig. 2).

II

Fig. 2 gibt die Abhängigkeit der Dissoziation der Aktin- und L-Myosinfilamente von der ATP Konzentration in Gegenwart konstanter Mengen der Interaktions-Inhibitoren. Die Abbildung zeigt, dass mit Erhöhung der ATP Konzentration nicht nur der Dehnungswiderstand sondern auch die aktive Spannung der extrahierten Fasern vollständig verschwindet. Die Abbildung zeigt ferner, dass unter den gleichen Bedingungen die Spaltung des ATP nicht vollständig verschwindet, sondern von der hohen Geschwindigkeit der Mg-aktivierten Aktomyosin-ATPase auf den niedrigen Wert der Mg-aktivierten L-Myosin-ATPase herabgesetzt wird.

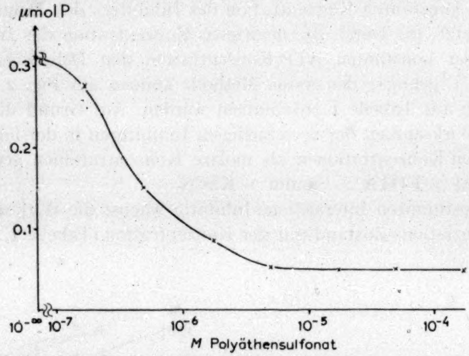

Fig. 3. Verwandlung der Aktomyosin-ATPase in L-Myosin-ATPase durch Polyäthensulfonat in Gegenwart von $3 \cdot 10^{-3}$ *M* ATP. Ordinate: Spaltungsgeschwindigkeit in µmol P/mg Fibrilleneiweiss × min. Abszisse: Molarität des Polyäthensulfonats. ($3 \cdot 10^{-3}$ *M* $MgCl_2$; 0.05 *M* KCl; 0.01 Tris–Maleinat-Puffer pH 6.9; Temperatur, 25°). Vor Zugabe von ATP werden die Fibrillen 10 min lang mit Polyäthensulfonat gerührt.

Auch wenn die Konzentration der Interaktions-Inhibitoren weit über die Konzentration erhöht wird, die der Fig. 2 zugrunde liegen, sinkt die Spaltungsgeschwindigkeit des ATP nicht weiter (d.h. nicht unter die Spaltungsrate der L-Myosin-ATPase). Dies ist für EDTA von PERRY UND GREY[11] sowie von BENDALL[12] und für Erschlaffungsfaktor von HASSELBACH (vergl. WEBER[3]) gezeigt. Für Polyäthensulfonat ergibt sich die gleiche Tatsache aus Fig. 3. Dieses Ergebnis ist zu erwarten weil alle Interaktions-Inhibitoren die Mg-aktivierte L-Myosin-ATPase in den Konzentrationen nicht beeinflussen, in denen sie auf die Mg-aktivierte Aktomyosin-ATPase voll wirksam sind (vgl. für Polyäthensulfonat BÁRÁNY UND BÁRÁNY[6], für Erschlaffungsfaktor WEBER[3] (nach Versuchen von NAGAI, MAKINOSE UND HASSELBACH[4]) und für EDTA Tabelle II).

Wenn die ATP-Konzentration so niedrig wird, dass die Aktin- und L-Myosinfilamente mehr und mehr assoziieren, wächst die ATP-Spaltung und die maximale isometrische Spannung trotz der Anwesenheit der Interaktions-Inhibitoren proportional mit dem Dehnungswiderstand auf den Normalwert von 100 %* an (Fig. 2).

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* Als Wert von 100 % wird hier die Spaltungsgeschwindigkeit und die Spannung in Gegenwart von $3 \cdot 10^{-3}$ *M* ATP ohne Inhibitor bezeichnet; 100 % Dehnungswiderstand ist der Dehnungswiderstand der starren Fasern ohne ATP und ohne Inhibitor.

TABELLE II

EINFLUSS VON EDTA AUF DIE Mg-AKTIVIERTE ATP-SPALTUNG DES L-MYOSINGELS
Mg 10^{-2}; μ, 0.05; pH, 7.0; Temperatur, 25°.

Molaritat von EDTA	*µmol P × mg L-Myosin^{-1} × min^{-1}*
0	0.021
$2.5 \cdot 10^{-4}$	0.020
$5.0 \cdot 10^{-4}$	0.019
$1.0 \cdot 10^{-3}$	0.022
$2.5 \cdot 10^{-3}$	0.019
$5.0 \cdot 10^{-3}$	0.019

Das bedeutet: Die Inhibitoren dieser Arbeit, zu der auch der "relaxing factor" gehört, stören die Interaktion von Aktin und L-Myosin ausschliesslich dadurch, dass sie das Aktomyosin in Gegenwart der experimentell üblichen und physiologischen ATP-Konzentration zur Dissoziation bringen.

Diese Deutung erklärt ein Phänomen, das für den "relaxing factor" schon seit langem bekannt ist und das auch in Anwesenheit der anderen Interaktions-Inhibitoren dieser Arbeit auftritt. Fasern die Interaktions-Inhibitoren enthalten, erschlaffen beim Einbringen in ausreichend konzentrierten ATP-Lösungen erst, nachdem sie sich zunächst flüchtig kontrahiert haben (Fig. 4). Dieses Verhalten ist auf Grund der mitgeteilten Beobachtungen zu erwarten. Denn unmittelbar nach dem Eintauchen in die ATP-Lösung wächst die ATP-Konzentration im Innern der Fasern von Null auf den Wert des stationären Gleichgewichts. Infolgedessen ist in den ersten Sekunden des Eintauchens die ATP-Konzentration "innen" so klein, dass Dissoziation und Hemmung noch nicht stattfinden (Kontraktion). Einige Sekunden später aber ist die ATP-Konzentration genügend angewachsen, so dass der Inhibitor wirksam wird (Dauer-Erschlaffung). Infolgedessen können Interaktions-Inhibitoren, die ausschliesslich durch Dissoziation des Aktomyosins wirksam werden, auch daran erkannt werden, dass sie eine flüchtige Anfangskontraktion der gehemmten extrahierten Fasern mit nachfolgender Dauererschlaffung hervorrufen.

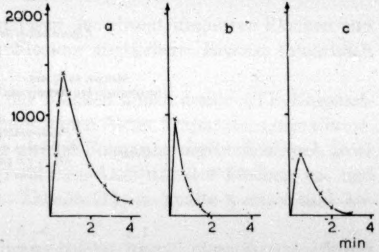

Fig. 4. Flüchtige Anfangskontraktion mit verschiedenen Interaktions-Inhibitoren inkubierten Einzelfasern beim Einbringen in die Inhibitor enthaltende ATP-Lösung. Ordinate: Isometrische Spannung in g/cm². Abszisse: Zeit in Minuten. (a) Erschlaffungsfaktor 2.0 mg/ml. (b) 10^{-3} *M* EDTA. (c) 10^{-4} *M* Polyäthensulfonat. ($3 \cdot 10^{-3}$ *M* ATP; $3 \cdot 10^{-3}$ *M* $MgCl_2$; 0.05 *M* KCl; 0.01 *M* Tris–Maleinat-Puffer pH 6.9; Temperatur, 25°. Faserdurchmesser = 40–50 μ; Anfangslänge = 80%).

III

Wenn in einer Faser die Kohäsionskräfte zwischen Aktin und L-Myosin völlig aufgehoben sind, sinkt zwar der Dehnungswiderstand auf Null, doch wird die strukturelle

TABELLE III

DIE ABGABE VON F-AKTIN AUS GEREINIGTEN AKTOMYOSIN-FLOCKEN IN GEGENWART VON POLYÄTHENSULFONAT (PÄS) UND ATP

$\mu = 0.05$; pH, 7.0; Mg, 10^{-3} *M*; Temperatur, 25°

Bedingungen der Aktinabgabe Konzentration der Suspension der AM-Flocken ≦ 0.5 mg/ml in Gegenwart einer Molarilat von		*A) Analyse der Flocken Eigenschaften der Eiweissflocken nach Abzentrifugieren und zweimaliger Umfallung*			
		ATP-Empfindlichkeit der Viskosität in % ($\mu = 0.5$)		*Mg-aktivierte ATP-Spaltung in µmol P × mg umgefälltes Eiweiss^{-1} × min^{-1} ($\mu = 0.05$) Nach Neutralisierung des PÄS**	
PÄS (1)	*ATP (2)*	*Ohne Zusatz (3)*	*Nach Zusatz von Aktinüberschuss (4)*	*Ohne Zusatz (5)*	*Nach Zusatz von Aktinüberschuss (6)*
—	—	139	164	0.36	0.49
$5.0 \cdot 10^{-7}$	—	133	161	0.38	0.48
$1.0 \cdot 10^{-6}$	—	135	162	0.33	0.42
$2.0 \cdot 10^{-6}$	—	131	160	0.27	0.38
—	$1.0 \cdot 10^{-3}$	120	163	0.30	0.50
$5.0 \cdot 10^{-7}$	$1.0 \cdot 10^{-3}$	59	159	0.18	0.49
$1.0 \cdot 10^{-6}$	$1.0 \cdot 10^{-3}$	35	155	0.12	0.39
$2.0 \cdot 10^{-6}$	$1.0 \cdot 10^{-3}$	31	148	0.08	0.31

B) Analyse des Überstandes

Eigenschaften des bei pH 4.7 gefällten Eiweisses des Überstandes

		Mengen in % des ursprünglichen Aktomyosin		*Eigenschaften der Eiweissfraktion aus Spalte (4)*				
				ATP-Empfindlichkeit in % bei 0.5 µ			*ATP-Spaltung** bei 0.05 µ*	
		Total (3)	*Proteinfraktion, die bei $\mu = 0.025$ pH = 7.0 gelöst bleibt (4)*	*Ohne Zusatz (5)*	*Mit Aktin (6)*	*Mit L-Myosin (7)*	*Ohne Zusatz (8)*	*Mit L-Myosin (9)*
—	—	1.5	~ 0					
$5 \cdot 10^{-7}$	—	3.6	0.7	—	—	6		
$1 \cdot 10^{-6}$	—	5.7	1.0	—	—	5		
$2 \cdot 10^{-6}$	—	6.6	0.8	—	—	5		
—	$1 \cdot 10^{-3}$	4.1	4.1	1	2	125	~ 0	0.30
$5 \cdot 10^{-7}$	$1 \cdot 10^{-3}$	14.2	11.9	0	2	109		
$1 \cdot 10^{-6}$	$1 \cdot 10^{-3}$	16.0	11.2	0	3	107		
$2 \cdot 10^{-6}$	$1 \cdot 10^{-3}$	17.3	11.4	2	4	97		

* Da die Eiweissflocken das Polyäthensulfonat sehr fest binden, stehen die abzentrifugierten und umgefällten Flocken immer noch unter Polyäthensulfonat-Wirkung. Diese Wirkung beeinflusst die ATP-Empfindlichkeit nicht (vergl. A, Spalte 3 und B, Spalte 7), aber vermindert die ATP-Spaltung. Durch Titration mit Protamin[6] kann diese Wirkung weitgehend, aber nicht ganz neutralisiert werden. Infolgedessen zeigt die ATP-Spaltung einen schwachen Gang in Abhängigkeit von der ursprünglichen Polyäthensulfonat-Konzentration (der Spalte 1) auch dann, wenn der Aktingehalt der Flocken normal ist (vergl. A, Spalte 6).

** Da hier auf die Neutralisierung verzichtet wurde, ist ein Spaltungswert ausschliesslich nach Vorbehandlung mit ATP ohne Polyäthensulfonat angeführt.

Anordnung der Filamente nicht gestört. Die Myosinfilamente bleiben am ihrem Platz, weil das L-Myosin bei der gegebenen Ionenstärke unlöslich ist. Die Aktinfilamente bleiben an ihrem Platz, weil sie durch ihre Befestigung an der Z-Scheibe fixiert sind. Diese mechanische Fixierung der Aktinfilamente fehlt, wenn der Aktomyosin-Komplex in der Form einer Flocke von hoch gereinigtem Aktomyosin vorhanden ist.

Infolgedessen ist es möglich, die Dissoziation von Aktin und L-Myosin auch analytisch nachzuweisen, falls Aktomyosin-Flocken bei niedriger Ionenstärke zur Dissoziation gebracht werden. Das L-Myosin bleibt in der Flocke, weil es unlöslich ist, und das Aktin diffundiert aus der Flocke heraus, weil es löslich ist und von dem L-Myosin Teilchen nicht länger gebunden wird.

Tabelle III zeigt, dass in Gegenwart von ATP und dem Interaktions-Inhibitor Polyäthensulfonat aus natürlichen Aktomyosin-Flocken der grösste Teil des Aktins, bei pH 7.0 und $\mu = 0.05$, austritt*. Die Aktin-Abgabe durch die Flocken unterbleibt, wenn allein Polyäthensulfonat ohne ATP anwesend ist. Aus der ATP Empfindlichkeit der Viskosität der abzentrifugierten mit 0.5 *M* KCl gelösten Flocken (vergl. Tabelle III; A, Spalte 3) errechnet sich ein Aktinverlust von etwa 80 % des ursprünglichen Aktingehaltes, falls mit $2 \cdot 10^{-6}$ *M* Polyäthensulfonat in Anwesenheit von 10^{-3} *M* ATP inkubiert wird. Ähnliche Werte ergeben sich aus der ATPase-Aktivität der Flocken bei 0.05 μ (vergl. Tabelle III; A, Spalte 5). Aus dem Überstand dieser Flocken kann dagegen nur eine Menge von etwa 50 % des ursprünglichen Aktin isoliert werden, wenn man den Aktingehalt des ursprünglichen Aktomyosins zu 25 % des Aktomyosins ansetzt. (Vergl. Tabelle III; B, Spalte 4)**.

Die in der Tabelle III enthaltenen Analysen der abzentrifugierten Flocken und ihres Überstandes beweisen, dass das von Flocken abgegebene Eiweiss tatsächlich Aktin ist.

1. Die mit wachsendem Aktinverlust der Flocken abnehmende ATP-Empfindlichkeit der Viskosität wird durch Zugabe eines reinen Aktin Präparates normalisiert. (Vergl. Tabelle III; A, Spalte 3 mit 4). Die gleiche Folgerung ergibt sich auch trotz einiger technischer Schwierigkeiten aus der ATPase-Aktivität der Flocken vor und nach Zusatz von gereinigtem Aktin. (Vergl. Tabelle III; A, Spalte 5 mit 6 und Anmerkung* zu der Tabelle III.)
2. Das aus dem Überstand isolierte Eiweiss besitzt sowohl ohne Zusatz wie mit Aktin-Zusatz praktisch keine ATP-Empfindlichkeit der Viskosität. Dagegen treten sofort ATP-Empfindlichkeiten über 100 % auf, wenn zu dem isolierten Eiweiss L-Myosin zugegeben wird. (Vergl. Tabelle III; B, Spalte 5 und 6 mit 7.)

Während das Polyäthensulfonat ohne ATP nicht zu einem Aktinverlust der Aktomyosin-Flocken führt (Tabelle III), vermag ATP sogar in der niedrigen Konzentration von $1 \cdot 10^{-3}$ *M*, auch in Abwesenheit von Interaktions-Inhibitoren, einen kleinen Teil der Aktin-Komponente durch Dissoziation in Lösung zu bringen. Wenn die Aktomyosin-Flocken aus der Aktomyosin-Lösung abzentrifugiert werden, bevor die ATP-Konzentration durch Spaltung deutlich abgenommen hat, ist die ATP-Empfindlichkeit der Viskosität der abgetrennten und in 0.5 M KCl gelösten Flocken und die ATPase-Aktivität um ungefähr 15 % erniedrigt (vergl. Tabelle III; A, Spalte 3 und Spalte 5). Auch diese geringfügigen Abnahmen verschwinden bei Zugabe eines Aktinüberschusses (vergl. Tabelle III; A, Spalte 3 mit 4 und 5 mit 6). Der dissoziierende ATP-Effekt wird deutlicher und einwandfrei nachgewiesen durch die Analyse des Überstandes. Nach Inkubation mit 10^{-3} M ATP wird aus dem Überstand Aktin in einer Menge von 4 % des ursprünglichen Aktomyosin oder in einer Menge von etwa 16 % der Aktin-Komponente isoliert. Dass dieses isolierte Protein tatsächlich aus Aktin besteht, ergibt sich durch L-Myosin-Zusatz nicht nur für die ATP-Empfindlichkeit der Viskosität, sondern auch für die ATPase-Aktivität (Tabelle III; B, Spalte 7 und 9)*.

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* HIPPEL, GELLER UND MORALES[13] vertreten die Ansicht, Myosin-B oder natürliches Aktomyosin sei kein Komplex aus Aktin und Myosin. Sie berufen sich darauf, dass es noch nie gelungen sei aus natürlichen Aktomyosin-Präparaten wesentliche Mengen an Aktin präparativ zu isolieren. Unsere Beobachtungen füllen diese Lücke und zeigen damit, dass natürliches Aktomyosin zu einem Viertel bis einem Fünftel aus Aktin besteht.

** Offensichtlich geht durch die Fällung der Überstand-Eiweisskörper bei pH 4.7 (vergl. Tabelle III; B, Spalte 3) und die darauf folgende Fällung der bei pH 7.0 und 0.025 μ unlöslichen Eiweisskörper ein gewisser Teil des Aktins verloren (vergl. Tabelle III; B, Spalte 4). Dies dürfte auch der Grund sein, aus dem die Aktin-Ausbeute im Überstand für ATP-haltige Ansätze mit $5 \cdot 10^{-7}$ *M* PÄS und $2 \cdot 10^{-6}$ *M* PÄS gleich ist, obwohl der Aktinverlust der Flocken mit der PÄS-Konzentration zunimmt. (Vergl. Tabelle III; B, Spalte 4 mit A, Spalte 3).

Da die Aktomyosin-Dissoziation in Gegenwart von 10^{-3} M ATP unvollständig ist, superpräcipitieren die Flocken im Gegensatz zu den Flocken, die mit 10^{-3} M ATP und Polyäthensulfonat inkubiert werden.

IV

Die Beobachtungen dieser Arbeit zeigen, dass die mechanochemische Interaktion zwischen Aktin- und L-Myosin—die die Kontraktion hervorruft—nicht mehr stattfindet, wenn die enzymatische Interaktion wegfällt durch die die Mg-aktivierte Aktomyosin-ATPase das ATP spaltet. Dieser Parallelismus zwischen mechanischer und enzymatischer Interaktion gilt sowohl für die Aktin- und L-Myosinfilamente der Fibrille, wie für die Aktin- und L-Myosin Moleküle des Gels aus natürlichem Aktomyosin. Es liegt nahe diesen Parallelismus energetisch zu erklären. Durch die Dissoziation des Aktomyosins-Systems in Anwesenheit von ATP und Interaktions-Inhibitor wird die Mg-aktivierte ATP-Spaltung und damit auch der Betrag der Spaltungsenergie auf 10% des Normalwertes herabgesetzt. Dieser Restbetrag an Energie ist aber sicher für eine normale Kontraktion zu klein.

Diese energetische Erklärung ist ungenügend. Denn eine extrahierte Faser, die durch Mg und ATP zur Kontraktion gebracht ist erschlafft vollständig, wenn sie in ein Bad übertragen wird, das Interaktions-Inhibitor, ATP und Ca enthält (Fig. 5). Gleichzeitig sinkt der Dehnungswiderstand auf Null. In Ca Gegenwart aber spaltet die dissoziierte Faser das ATP nicht langsamer sondern etwa doppelt so schnell wie vorher während der Kontraktion (Fig. 5). Das ist selbstverständlich, weil die L-Myosin-ATPase der dissoziierten Fasern durch Ca ausserordentlich aktiviert wird. Trotz dieser starken ATP-Spaltung tritt Erschlaffung ein, weil das ATP nicht durch enzymatische Interaktion von Aktin- und L-Myosin sondern ausschliesslich durch die L-Myosin-ATPase gespalten wird.

H. H. WEBER[3] hat auf Grund von Beobachtungen über die Wirkung des Erschlaffungsfaktors die Meinung vertreten, dass ATP-Spaltung nur dann Kontraktion hervorruft, wenn diese Spaltung auf eine Interaktion zwischen Aktin und L-Myosin beruht. Diese Ansicht ist durch die Analyse der chemisch bekannten Interaktions-

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* Der Nachweis, dass 10^{-3} M ATP allein selbst bei der niedrigen Ionenstärke von 0.05 einen schwachen, aber deutlichen Dissoziationseffekt auf Aktomyosin ausübt, passt zu der Beobachtung von HASSELBACH *et al.*[14], dass bei der Ionenstärke 0.25 μ durch $7 \cdot 10^{-3}$ M ATP aus Muskelfibrillen etwas L-Myosin ausgelöst wird.

Inhibitoren in dieser Arbeit auf eine breite Grundlage gestellt. Darüber hinaus ist gezeigt, dass eine ATP-Spaltung durch die L-Myosin-ATPase selbst dann keine Kontraktion hervorruft, wenn diese Spaltung viel grösser ist als die ATP-Spaltung durch die normale Mg-aktivierte Fibrille die zur maximalen Kontraktion führt.

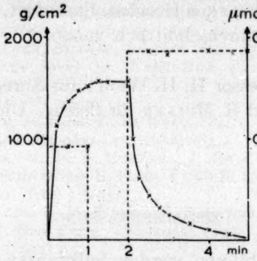

Fig. 5. Vergleich der isometrischen Spannung und der ATP-Spaltung beim Übergang aus einer Polyäthensulfonat freien Mg-haltigen ATP-Lösung in eine Polyäthensulfonate- und Ca-haltige ATP-Lösung. Abszisse: Zeit in Minuten. Linke Ordinate: Isometrische Spannung in g/cm². Rechte Ordinate: Spaltungsgeschwindigkeit des ATP in μmol P/mg Fibrilleneiweiss × min. ×—×, isometrische Spannung; ×---×, ATP-Spaltung. Erste Minute: Kontraktion mit $3 \cdot 10^{-3}$ *M* ATP und $3 \cdot 10^{-3}$ *M* Mg. Zweite Minute: Auswaschen mit KCl und Puffer. Dritte bis fünfte Minute: Erschlaffung mit $5 \cdot 10^{-3}$ *M* ATP, 0.01 *M* $CaCl_2$ und $1 \cdot 10^{-4}$ *M* Polyäthensulfonat; (0.05 *M* KCl; 0.01 *M* Tris–Maleinat-Puffer pH 6.9; Temperatur, 25°; Einzelfaser = 40 μ; Anfangs-Länge = 40 %). Erklärung siehe im Text.

METHODISCHER TEIL

Die Messung der Spannungsentwicklung und des Dehnungswiderstandes erfolgt mit dem von A. Weber[15] angegebenen Tensiometer.

Die Glyzerin-extrahierten Fasern aus dem *M. psoas* werden ebenfalls nach dem Verfahren von A. Weber[15] hergestellt. Ihr Durchmesser wird mit dem Leitz-Objektiv U.M.3 20 × gemessen. Der Faserdurchmesser beträgt 35–65 μ, im Mittel 50 μ.

Die Herstellung der Fibrillen erfolgt wie bei Ulbrecht und Ulbrecht[7], die des natürlichen Aktomyosins wie bei Bárány und Bárány[5], die des L-Myosins wie bei Portzehl, Schramm und Weber[16] und die des Aktins wie bei Mommaerts[17].

Die ATP-Empfindlichkeit wird mit der Formel von Portzehl, Schramm und Weber[16] gerechnet. Die Strukturviskosität der Aktomyosinlösungen wird nach Jaisle[18] in Betracht gezogen.

Bei der ATPase Bestimmung werden die Ansätze magnetisch gerührt. Es wird darauf geachtet, dass während der Spaltungszeit nicht mehr als 25 % des zugesetzten ATP gespalten werden. Das freigesetzte anorganische Phosphat wird nach Rockstein und Herron[19], in Gegenwart von KSCN nach Fiske und Subbarow[20], gemessen. In Gegenwart von Fuadin werden von dem Trichloressigsäure-Filtrat so kleine Mengen zur Phosphatbestimmung genommen, dass der Antimongehalt des Fuadin die Bestimmung noch nicht stört. Bei ATP-Konzentrationen unter $1 \cdot 10^{-3}$ *M* wird die ATPase-Aktivität mit der optischen Methode von Fischer und Siebert[21] bestimmt, dabei wird das gebildete ADP durch Pyruvatkinase umgesetzt und die auftretende Brenztraubensäure durch DPNH-Oxidation mittels Lacticodehydrogenase erfasst.

Erschlaffungsfaktor wird nach der Methode von PORTZEHL[22] hergestellt, wobei vorwiegend dialysierte Granasuspension verwandt wird.

Pyruvatkinase, Lacticodehydrogenase, DPNH und Phospho-enol-Brenztraubensäure stammen von der Firma Boehringer und Soehne, Mannheim, das ATP von der Firma Pabst, Milwaukee. Für die freundliche Überlassung von Polyäthensulfosaurem Natrium danken wir den Farbwerken Hoechst, Frankfurt, ebenso wie für die Angaben des Molekulargewichtes von durchschnittlich 30000.

Wir danken Herrn Professor H. H. WEBER für Anregung und fördernde Diskussionen, Fräulein G. BÖKH und R. MÜLLER für fleissige Unterstützung bei der Durchführung der Experimente.

ZUSAMMENFASSUNG

1. Als Interaktions-Inhibitoren werden Substanzen bezeichnet, die die ATP-Spaltung durch L-Myosin unter keinen Bedingungen beeinflussen, aber in Gegenwart von ATP die enzymatische Interaktion zwischen Aktin und L-Myosin aufheben, *i.e.* sie verwandeln die Mg-aktivierte Aktomyosin-ATPase reversibel in L-Myosin-ATPase.

2. Der Erschlaffungsfaktor von MARSH UND BENDALL, einige Polykationen sowie Polysulfonate, EDTA und SCN^- in niedrigen Konzentrationen sind Interaktions-Inhibitoren.

3. Es wird für Polyäthensulfonat, EDTA, Fuadin und SCN^- gezeigt und für den Erschlaffungsfaktor bestätigt, dass diese Substanzen in ATP-Gegenwart zusammen mit der chemischen Interaktion auch die Assoziation von Aktin und L-Myosin reversibel aufheben.

4. Dies wird für die Fibrillen in den Wasser-Glycerin-extrahierten Fasern durch Messung des Dehnungswiderstandes festgestellt.

5. Für Gele aus natürlichem Aktomyosin ergibt sich der gleiche Schluss daraus, dass aus diesen Gelen die Aktin-Komponente durch eine ATP-haltige Polyäthensulfonatlösung fast vollständig extrahiert werden kann.

6. Kontrahierte Wasser-Glycerin-extrahierte Fasern erschlaffen, sobald die Aktin- und L-Myosin-Filamente durch die Wirkung von ATP und Interaktions-Inhibitor dissoziieren.

7. Die Dissoziation von Aktin und L-Myosin verschwindet, wenn entweder die Konzentration des ATP oder des Interaktions-Inhibitors genügend herabgesetzt wird.

8. In beiden Fällen tritt zusammen mit der wiedererscheinenden Assoziation auch Kontraktion und ATP-Spaltung durch Mg-aktivierte Aktomyosin-ATPase von neuem auf.

9. Die Erschlaffung unter ATP und Interaktions-Inhibitor tritt nicht nur dann auf, wenn die Mg-aktivierte Aktomyosin-ATPase in eine wenig wirksame Mg-aktivierte L-Myosin-ATPase verwandelt wird, sondern auch bei Verwandlung in die hochwirksame Ca-aktivierte L-Myosin-ATPase. Die Kontraktion wird ausschliesslich hervorgerufen, wenn der Spaltungsmechanismus des ATP auf der Interaktion zwischen Aktin und L-Myosin beruht. Die Kontraktion ist unabhängig von der ATP-Spaltung durch L-Myosin-ATPase.

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POLYELEKTROLYTE ALS INTERAKTIONS-INHIBITOREN UND DIE BEDEUTUNG VON Ca UND Mg FÜR DIE AKTIN–MYOSIN INTERAKTION

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SUMMARY

Polyelectrolytes as interaction inhibitors and the significance of Ca and Mg in actin–myosin interaction

1. Evidence is given that there are interaction inhibitors as well as myosin ATPase poisons among the high molecular electrolytes.

2. It is shown that Ca-activated actomyosin in both the gel and the sol state splits ATP quantitatively with the same speed with which pure L-myosin under the same conditions splits ATP if the splitting by actomyosin is related to the unit of weight of the L-myosin component that is contained in the actomyosin.

3. The rate of Ca-activated ATP splitting is likewise identical in actomyosin and L-myosin systems if myosin ATPase poisons or interaction inhibitors are present. In the presence of myosin ATPase poisons the splitting in both systems decreases to the same extent. In the presence of interaction inhibitors the splitting in both systems is not affected at all.

The observations 2 and 3 lead to the conclusion that the Ca-activated ATP splitting—even in actomyosin gels—is always based on the action of the L-myosin ATPase. Only the presence of Mg brings about in actomyosin gels an enzymic interaction between actin and myosin.

4. The interaction inhibition by protamine and polyethensulfonate is not based on an increase in the ionic strength and is independent of the protein charge in the pH range from 6 to 9.

5. The interaction inhibitions by means of the polyanion polyanetholsulfonate and the polycation protamine can be shown to be the same for all concentrations of polyions if the inhibition is related to the amount of polyions that is bound by actomyosin instead of to the amount of polyions added.

6. The polycation protamine is preponderantly bound by F-actin, whereas the polyanion polyethensulfonate is almost exclusively bound by L-myosin.

7. When high polymer interaction inhibitors are joined with contractile proteins they dissociate less rapidly and completely than do low molecular inhibitors and the proteins. It is shown that also the binding of polyelectrolytes to actomyosin is reversible.

EINLEITUNG

Die Tatsache, dass sogenannte Interaktions-Inhibitoren (siehe die voranstehende Arbeit[1]) in Gegenwart von ATP alle Interaktionen zwischen Aktin und L-Myosin in extrahierten Fasern, Fibrillen und Aktomyosingelen vollständig aufheben, lässt eine weitere Analyse lohnend erscheinen.

Infolgedessen werden in der vorliegenden Arbeit eine Anzahl hochpolymeren Elektrolyte daraufhin untersucht, wieweit sie ATP-Spaltung durch Interaktionshemmung oder durch Beeinflussung des L-Myosin hemmen.

I

Nicht alle Elektrolyte mit einem umfangreichen Kohlenstoffskelett sind Interaktions-Inhibitoren. Sowohl grosse organische Kationen wie Cetyltrimethylammonium

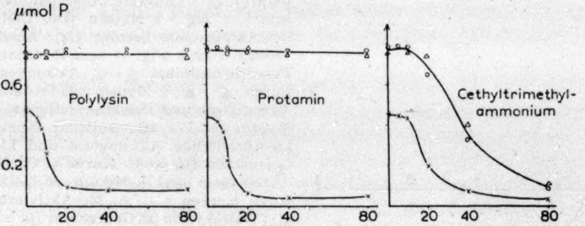

Fig. 1. Die ATP-Spaltung durch natürliches Aktomyosingel und L-Myosingel unter dem Einfluss von grossen organischen Kationen (μ, 0.05 – 0.08; pH, 7.0; Temperatur, 25°). Ordinate: Spaltungsgeschwindigkeit in μmol. P $\times$ min^{-1} $\times$ mg^{-1}. Abszisse: Äquivalente des hochmolekularen Kations. Die Zahl der Äquivalente bedeutet die Zahl der Ladungen des Inhibitors die zu 10^5 g Eiweiss hinzugefügt sind. ×—×, Mg-aktiviertes Aktomyosingel; ○—○, Ca-aktiviertes Aktomyosingel (Spaltung bezogen auf den L-Myosingehalt des Gels); △—△, Ca-aktiviertes L-Myosingel.

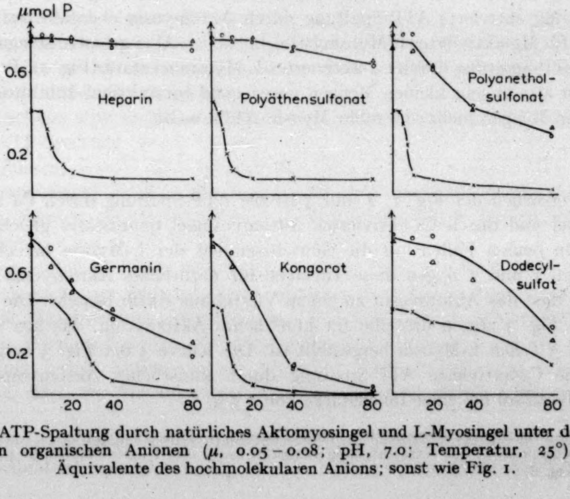

Fig. 2. Die ATP-Spaltung durch natürliches Aktomyosingel und L-Myosingel unter dem Einfluss von grossen organischen Anionen (μ, 0.05 – 0.08; pH, 7.0; Temperatur, 25°). Abszisse: Äquivalente des hochmolekularen Anions; sonst wie Fig. 1.

(Fig. 1) als auch grosse organische Anionen wie Germanin*, Kongorot** und Dodecylsulfat (Fig. 2) erniedrigen die ATP-Spaltung durch L-Myosingel ebenso stark wie die Spaltung durch Mg-aktiviertes Aktomyosingel. Dagegen sind die polymeren Kationen Polylysin (vergl. BÁRÁNY UND BÁRÁNY[2]) und Protamin (Fig. 1) und die polymeren Anionen Heparin und Polyäthensulfonat typische Interaktions-Inhibitoren (Fig. 2). Sie beeinflussen die ATP-Spaltung durch L-Myosingel nicht, (Fig. 1 und 2) aber verwandeln die Mg-aktivierte Aktomyosin-ATPase reversibel*** in die L-Myosin-ATPase, (Fig. 1, 2 und Kurven 4, 5, 6 der Fig. 3) falls die Inhibitor-Konzentration hoch genug ist. Eine weitere Erhöhung der Konzentration erhöht die ATPase Hemmung nicht weiter (Fig. 1, 2 und Kurven 4, 5, 6 der Fig. 3). Eine Zwischenstellung nimmt das Polyanetholsulfonat ein, sehr kleine Mengen dieses Inhibitors setzen die Mg aktivierte ATP-Spaltung durch Aktomyosin ebenfalls auf den Wert herab, der für Mg-aktiviertes L-Myosin bezeichnend ist. Aber grössere Mengen hemmen auch die ATP-Spaltung durch Ca-aktiviertes L-Myosingel stark (Fig. 2). Polyanetholsulfonat ist also in sehr kleinen Mengen vorwiegend Interaktions-Inhibitor und wird in grösseren Mengen mehr und mehr Myosin-ATPase-Gift.

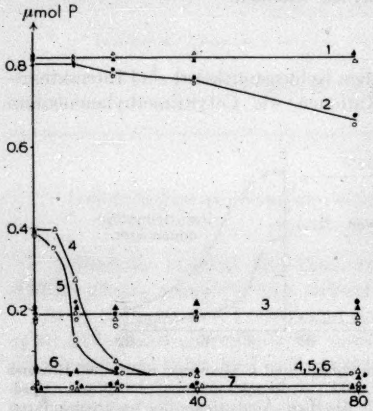

Fig. 3. Vergleich der Ca- und Mg-aktivierten ATP-Spaltung durch künstliches Aktomyosin und durch L-Myosin unter dem Einfluss von Protamin und Polyäthensulfonat (pH, 7.0; Temperatur, 25°). Ordinate: Abgespaltenes μmol P × $\min^{-1}$ × mg^{-1} L-Myosin. (Der L-Myosingehalt des Aktomyosins beträgt 3/4). Abszisse: Äquivalente (vergl. Fig. 1) von Protamin bzw. von Polyäthensulfonat. △—△, Aktomyosin und Protamin; ▲—▲, L-Myosin und Protamin; ○—○, Aktomyosin und Polyäthensulfonat; ●—●, L-Myosin und Polyäthensulfonat. Kurven 1 und 2: Ca-Aktivierung Aktomyosin und L-Myosin im Gelzustand (μ, 0.08). Kurve 3: Ca-Aktivierung, Aktomyosin und L-Myosin im Solzustand (μ, 0.55). Kurven 4, 5, 6: Mg-Aktivierung; Kurve 4, 5 Aktomyosin im Gelzustand (μ, 0.05); Kurve 6 L-Myosin im Gelzustand (μ, 0.05). Kurve 7: Mg-Aktivierung, Aktomyosin und L-Myosin im Solzustand (μ, 0.52).

II

In allen Versuchen der Fig. 1, 2 und 3 ist die ATP-Spaltung durch Ca aktiviertes L-Myosingel und durch Ca-aktiviertes Aktomyosingel quantitativ gleich, falls die Spaltung in beiden Fällen auf die Gewichtseinheit des L-Myosin berechnet wird. Die Figuren 1 und 2 zeigen diese Tatsache für natürliches Aktomyosin unter der Annahme, dass das Aktomyosin zu einem Viertel aus Aktin besteht. Die Kurven 1 und 2 der Fig. 3 zeigen dasselbe für künstliches Aktomyosin, das aus einem Teil Aktin und 3 Teilen L-Myosin hergestellt ist. Die Kurve 3 der Fig. 3 vergleicht zusätzlich die Ca-aktivierte ATP-Spaltung durch künstliches Aktomyonsinsol und durch L-Myosinsol bei einer Ionenstärke von 0.5 μ.

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* Na -bis(-aminobenzol aminomethyl-benzoyl-naphthyl-aminotrisulfonat).
** Kupplungsprodukt aus tetrazotiertem Benzidin mit 2 Mol 1-aminonaphtalin-4-Sulfonsäure
*** Siehe Fig. 8.

Die Identität der Ca aktivierten Aktomyosin- und L-Myosin-ATPase geht aus folgenden Ergebnissen der Figuren 1–3 hervor.

1. Wenn kein Inhibitor anwesend ist spalten natürliches (Figuren 1 und 2) und künstliches (Fig. 3 Kurven 1, 2) Aktomyosingel einerseits, und L-Myosingel andrerseits ATP mit der gleichen hohen Spaltungsrate von 0.7–0.8 μ mol/mg L-Myosin × min.

2. Im Solzustand bei einer Ionenstärke von 0.5 μ spalten künstliches Aktomyosinsol und L-Myosinsol das ATP ebenfalls mit gleicher Rate aber mit der viel kleineren Geschwindigkeit von etwa 0.2 μ mol/mg L-Myosin × min.

3. Substanzen, die als Inhibitoren der L-Myosin-ATPase die ATP-Spaltung durch L-Myosingel mit steigender Konzentration mehr und mehr hemmen, hemmen auch die ATP-Spaltung durch Aktomyosingel in genau dem gleichen Umfang. Dies gilt für natürliches Aktomyosingel (Fig. 1: Cetyltrimethylammonium, Fig. 2: Germanin, Kongorot, Dodecylsulfat, sowie höhere Konzentrationen von Polyanetholsulfonat und Polyäthensulfonat) ebenso wie für künstliches Aktomyosin (Kurve 2 der Fig. 3).

4. Wenn dagegen Substanzen hinzugegeben werden, die als Interaktions-Inhibitor die ATP-Spaltung durch L-Myosin überhaupt nicht oder in der angewendeten Konzentration nicht beeinflussen, so beeinflussen diese Substanzen auch die Spaltung durch Ca-aktiviertes Aktomyosin nicht. Das zeigen für natürliches Aktomyosingel die Fig. 1 (Polylysin, Protamin) und die Fig. 2 (Heparin sowie niedrige Konzentrationen an Polyäthensulfonat und Polyanetholsulfonat). Für künstliches Aktomyosingel wird dasselbe durch Kurve 1 und dem Anfangsteil der Kurve 2 der Fig. 3 demonstriert. Für L-Myosin und Aktomyosin im Solzustand ergibt sich die Einflusslosigkeit von Protamin und Polyäthensulfonat aus der Kurve 3 der Fig. 3.

Die aufgeführten Befunde (1–3) zeigen, dass die Anwesenheit von Aktin die Geschwindigkeit der Ca-aktivierten ATP-Spaltung durch die L-Myosin Komponente nie beeinflusst. Dieser Befund ist unabhängig davon, ob die Ca-aktivierte ATP-Spaltung durch die L-Myosin Komponente in Abhängigkeit von Ionenstärke und Giften, gross oder klein ist. Das bedeutet, dass die ATP-Spaltung unter Aktivierung durch Ca weder im Aktomyosingel noch im Aktomyosinsol auf einer Interaktion zwischen Aktin und L-Myosin beruht.

Dieser Schluss wird bestätigt durch die Beobachtung (4), nach der Interaktions-Gifte die ATP-Spaltung durch Ca-aktiviertes Aktomyosinsol und Aktomyosingel nicht beeinflussen, obwohl diese Interaktions-Gifte die Mg-aktivierte ATP-Spaltung durch Aktomyosingel in eine Mg beeinflusste ATP-Spaltung durch L-Myosingel verwandeln (Kurven der Mg-aktivierten ATP-Spaltung durch Interaktions-Inhibitoren in Figuren 1 und 2 und Kurven 4, 5, 6 in Fig. 3).

Dagegen wirken die Interaktionsgifte auch im Falle der Mg-Aktivierung nicht, wenn es sich nicht um Aktomyosingel, sondern um Aktomyosinsol handelt. Dies zeigt die für Mg-aktiviertes Aktomyosinsol und L-Myosinsol gleiche Kurve 7 der Fig. 3. Die Wirkungslosigkeit der Interaktionsinhibitoren auf die Mg aktivierte ATP-Spaltung durch Aktomyosinsole ist nicht überraschend, weil schon länger bekannt ist, dass im Aktomyosinsol die ATP-Spaltung durch L-Myosin-ATPase erfolgt.

Die Ergebnisse dieser Arbeit vereinigen sich mit allen Erfahrungen der Literatur über die Bedeutung von Ca und Mg für die ATP-Spaltung durch Aktomyosin und

L-Myosin in folgender Schlussfolgerung: Ca aktiviert die ATP-Spaltung sowohl durch Aktomyosingel wie durch Aktomyosinsol deshalb, weil es bei beiden Systemen ausschliesslich die L-Myosin-ATPase aktiviert. Mg hemmt die ATP-Spaltung durch Aktomyosinsol, weil es die L-Myosin ATPase hemmt. Es aktiviert die ATP-Spaltung durch Aktomyosingel, weil es die Interaktion zwischen Aktin und L-Myosin ermöglicht, durch die L-Myosin-ATPase zu Aktomyosin-ATPase wird.

Diese Schlussfolgerung bietet eine Erklärung für die lange bekannte Tatsache, dass die Ca-Aktivierung von Aktomyosinsystemen nicht zur Kontraktion und Superpräzipitation führt. Denn nur die ATP-Spaltung durch Aktomyosin-ATPase d.h. durch Interaktion von Aktin und Myosin bewirkt Kontraktion.

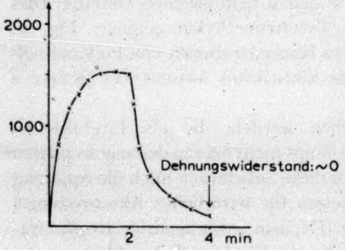

Fig. 4. Die Aktomyosin Dissoziation in der Wasser-Glyzerin extrahierten Einzelfaser in Gegenwart von 10^{-2} *M* ATP und 10^{-2} *M* Ca (pH, 6.9; Temperatur, 20°; Faserdicke, 30 μ; Anfangslänge, 35%). Ordinate: Isometrische Spannung in g/cm². Abszisse: Zeit in Minuten. Erste Minute: Kontraktion durch $3 \cdot 10^{-3}$ *M* ATP und Mg, 0.05 *M* KCl und 0.01 *M* Tris–Maleinat-Puffer. Zweite Minute: Waschen mit 0.05 *M* KCl und 0.01 *M* Tris–Maleinat-Puffer. Dritte und vierte Minute: Erschlaffung durch 10^{2} *M* ATP und Ca, 0.05 *M* KCl und 0.01 *M* Tris–Maleinat-Puffer. Senkrechter Pfeil Bestimmung des Dehnungswiderstandes in: $\mathrm{g/cm^2} \times L \times \Delta L^{-1}$.

Die Behauptung, durch Ca werde in Aktomyosingelen nur die L-Myosin-ATPase aktiviert, ist nicht identisch mit der Behauptung, durch Ca und ATP würden Aktomyosingele in ähnlicher Weise zur Dissoziation gebracht wie durch Interaktions-Inhibitoren und ATP. Es ist wahrscheinlich, dass in Ca aktivierten Aktomyosingelen einfach die Aktomyosin-ATPase aus Mg-Mangel nicht wirkt, während die L-Myosin-ATPase durch Ca spezifisch aktiviert wird. Da ohne Aktion der Aktomyosin-ATPase keine Kontraktion stattfindet, bewirkt das anwesende ATP durch seine Weichmacherwirkung auf jeden Fall eine gewisse Dissoziation des Aktomyosin. Diese Dissoziation kann sogar vollständig oder nahezu vollständig sein, wenn die ATP Konzentration sehr hoch ist (Fig. 4). Ob diese Dissoziation unter ATP und Ca für alle ATP Konzentrationen ebenso hoch ist, wie in Anwesenheit von ATP zusammen mit Interaktions-Inhibitoren, ist zur Zeit unbekannt.

III

Die Interaktionshemmung durch hochmolekulare Polyelektrolyte beruht nicht auf einer Erhöhung der Ionenstärke, wie man auf Grund der hohen Wertigkeitszahlen —34 Protamin*, 205 Polyäthensulfonat*—vermuten könnte. Denn die angewendeten Konzentrationen der Inhibitoren sind ausserordentlich niedrig. Auch in maximaler Konzentration liefert Protamin (berechnet nach der Formel $\mu = 1/2 \Sigma c \cdot z^2$) nur einen Beitrag von $1 \cdot 10^{-3}\ \mu$ und Polyäthensulfonat ein Beitrag von $5 \cdot 10^{-3}\ \mu$ zur Ionenstärke. Dieser Zuwachs durch die Inhibitoren fällt bei der gesamten Ionenstärke von $5 \cdot 10^{-2}\ \mu$ nicht ins Gewicht.

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* Diese Werte ergeben sich aus der chemischen Formel und dem Molekulargewicht von 7000 für Protamin[9] und von 30000 für Polyäthensulfonat (Mitteilung der Farbwerke Hoechst, Frankfurt).

Es ist schwierig anzunehmen, dass die Wirkung der Inhibitoren auf die Interaktion von Aktin und L-Myosin auf ihrer elektrischen Ladung beruht. Denn Polykationen und Polyanionen wirken gleichartig. Dagegen ist es sicher, dass die Wirkung der Interaktions-Inhibitoren unabhängig ist von den Ladungsänderungen des Aktomyosins zwischen pH 6 und 9. Falls man eine Konzentration an Inhibitor und ATP wählt, die bei pH 7.0 die ATP-Spaltung durch Mg aktiviertes Aktomyosin auf etwa die Hälfte herabsetzt, findet man eine proportionale Herabsetzung im ganzen pH-Bereich von pH 6 bis pH 9 (vergl. Fig. 5).

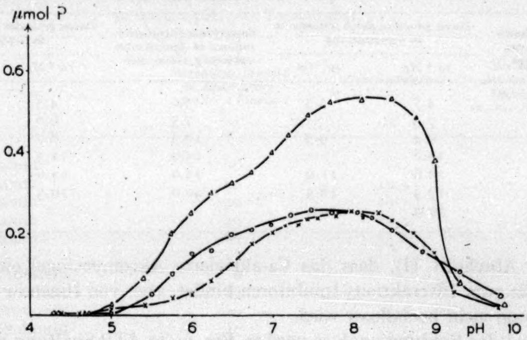

Fig. 5. Abhängigkeit des Einflusses von Protamin und Polyäthensulfonat vom pH der ATP Spaltung durch Mg-aktiviertes, natürliches Aktomyosingel (μ, 0.05; Temperatur, 25°). Ordinate: Spaltungsgeschwindigkeit in μmol P $\times$ min^{-1} $\times$ mg^{-1}. Abszisse: pH. △—△, ohne Interaktions-Inhibitor; ○—○, in Gegenwart von 7 Äquivalenten (vergl. Fig. 1) Polyäthensulfonat; ×—×, in Gegenwart von 10 Äquivalenten (vergl. Fig. 1) Protamin. Puffer, pH 4.0–5.5 Acetat; pH 5.7–7.5 Histidin; pH 7.5–8.9 Trisoxymethylaminomethan-HCl; pH 9.0–10 Glycin. Das pH wird im Ansatz gemessen.

IV

Die vom Aktomyosin gebundene Menge an Interaktions-Inhibitor kann direkt bestimmt werden, wenn man als Polykation Protamin und als Polyanion Polyanetholsulfonat anstelle von Polyäthensulfonat verwendet (vergl. METHODISCHER TEIL). Polyanetholsulfonat wirkt nach Abschnitt I dieser Arbeit allerdings nur bis zu einer Menge von 15 Äquivalenten/10^5 g Aktomyosin als praktisch reiner Interaktions-Inhibitor. In höheren Konzentrationen kommt zu der Hemmung der Interaktion auch eine Hemmung der Myosin-ATPase hinzu.

Tabelle I zeigt, dass bei Zugabe von 15 Äquivalenten von Polyanetholsulfonat zu 10^5 g Aktomyosin etwa 12 Äquivalente gebunden* werden. Polyanetholsulfonat erzeugt also eine reine Interaktions-Hemmung, solange nicht mehr als 12 Äquivalente durch 10^5 g Aktomyosin gebunden sind.

Die Bildung von 12 Äquivalenten an Protamin wird erst erreicht, wenn 25 Äquivalente zu 10^5 g Aktomyosin hinzugefügt werden. Die Affinität des Aktomyosins zu Polyanetholsulfonat ist also grösser als die Affinität zum Protamin. Tabelle I zeigt ferner, dass die Bindung beider Interaktions-Inhibitoren in Ca und Mg Gegenwart gleich ist. Die Wirkungslosigkeit der Interaktions-Inhibitoren auf die ATP-Spaltung durch Ca-aktiviertes Aktomyosingel beruht also nicht auf fehlender Bindung an das Aktomyosin, (Kurve 1a und 1b der Fig. 6). Dies ist ein weiteres Argument dafür (vergl. Abschnitt II), dass das Ca-aktivierte Aktomyosingel eine L-Myosin ATPase ist, die zwar Interaktions-Inhibitoren bindet, aber von Ihnen in der enzymatischen Wirkung nicht beeinflusst wird.

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* Die Bezeichnung der gebundenen Äquivalente bedeutet, dass pro 100000 g Eiweiss die angegebene Zahl von Grammäquivalenten oder Monomeren der betreffenden Substanzen am Inhibitormolekül fixiert ist. Die Bezeichnung bedeutet selbstverständlich nicht, dass jedes einzelne Monomer des Polymerinhibitors mit einer Gruppe des Eiweisses in Wechselwirkung steht. Wieviele der einzelnen Monomere in unmittelbarer Wechselwirkung mit dem Eiweiss stehen, bleibt völlig offen.

TABELLE I

DIE BINDUNG VON PROTAMIN UND POLYANETHOLSULFONAT AN AKTOMYOSIN

μ, 0.05; pH, 7.0; Temperatur, 25°.

Zugegebenes Protamin in Äquivalenten pro 10^5 g Aktomyosin	*Davon gebunden durch Aktomyosin in Gegenwart von*		*Zugegebenes Polyanetholsulfonat in Äquivalenten pro 10^5 g Aktomyosin*	*Davon gebunden durch Aktomyosin in Gegenwart von*	
	10^{-3} Mg	*10^{-2} Ca*		*10^{-3} Mg*	*10^{-2} Ca*
7.5	4.7	5.3	5.0	4.5	—
10.0	7.3	—	7.5	6.8	6.2
15.0	9.4	9.5	10.5	8.7	8.6
20.0	10.8	—	15.0	11.3	12.0
25.0	12.6	11.9	25.0	14.6	15.9
35.0	17.3	18.5	40.0	19.5	20.1
50.0	20.9	20.3			

Auf Grund der Bindungsanalyse wird in Fig. 6 die ATP-Spaltung in Abhängigkeit von den gebundenen Mengen an Protamin und Polyanetholsulfonat dargestellt. Die Darstellung führt nur bis zu der für Polyanetholsulfonat kritischen (siehe oben) Menge von 12 gebundenen Äquivalenten/10^5 g Aktomyosin. Die Abbildung zeigt, dass in Gegenwart von Mg die Interaktionshemmungen durch Protamin und durch Polyanetholsulfonat gleich sind, wenn die gebundenen Mengen beider Substanzen gleich sind, (vergl. Kurve 2a und 2b der Fig. 6). Die stärkere Wirkung des zugegebenen Polyanetholsulfonats im Vergleich mit Protamin (vergl. Fig. 1 mit 2) beruht also ausschliesslich auf seiner stärkeren Bindung durch Aktomyosin.

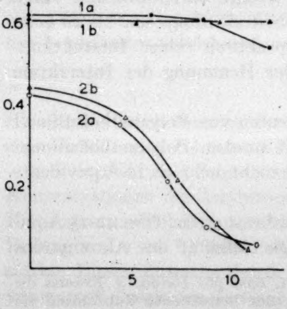

Fig. 6. Die Abhängigkeit der Ca- und Mg-aktivierten ATP-Spaltung durch natürliches Aktomyosin von der gebundenen Menge an Protamin oder Polyanetholsulfonat (μ, 0.05; pH, 7.0; Temperatur, 25°). Ordinate: Spaltungsgeschwindigkeit in μmol P × min^{-1} × mg^{-1}. Abszisse: Gebundene Äquivalente (vergl. dazu Anmerkung im Text). Volle Symbole, Aktivierung durch Ca. Leere Symbole, Aktivierung durch Mg; Dreiecke, Abhängigkeit von gebundenem Protamin; Kreise, Abhängigkeit von gebundenem Polyanetholsulfonat.

V

Der Betrag der Bindung von Protamin und Polyäthensulfonat durch Aktin einerseits und durch L-Myosin andererseits kann aus Tabelle II abgelesen werden. In Spalte 3 der Tabelle II sind die direkt bestimmten Werte angegeben (siehe METHODISCHER TEIL). In Spalte 4 finden sich die Raten der Mg aktivierten ATP-Spaltung, die auftreten, wenn das Aktin bzw. L-Myosin aus der Inhibitor-haltigen Lösung abzentrifugiert sind und durch die andere Aktomyosin Komponente (im Verhältnis 2.5 L-Myosin + 1.0 Aktin) in Aktomyosin verwandelt werden. Aus den ATPase-Hemmungen der Spalte 4 kann die Menge an Inhibitor roh abgeschätzt werden, die die Aktin- oder L-Myosin-Komponente in das Aktomyosin hineingeschleppt hat. Auf diesem indirekten Weg lässt sich auch die Polyäthensulfonat-Menge abschätzen, die durch Aktin oder L-Myosin gebunden und so in das Aktomyosin eingeführt wird. Es zeigt sich für Protamin übereinstimmend in Spalte 3 und Spalte 4, dass das Protamin von Aktin viel stärker gebunden wird als von L-Myosin. Für Polyäthensulfonat ergibt sich aus der Spalte 4 der Tabelle II, dass hier die Verhältnisse umgekehrt liegen.

TABELLE II

DIE BINDUNG VON PROTAMIN UND POLYÄTHENSULFONAT AN AKTIN UND L-MYOSIN

μ, 0.05; pH, 7.0; Temperatur, 25°.

Behandlung (1)	*Zugegebener Interaktions-Inhibitor in Äquivalenten pro 10^5 g Eiweiss* (2)	*Davon gebunden* (3)	*ATPase-Aktivitat des künstlichen AM, das aus dem behandelten Eiweiss hergestellt ist, in µmol P × min^{-1} × mg^{-1}* (4)
Aktin mit Protamin	0	—	0.35
Aktin mit Protamin	10	10.3	0.09
Aktin mit Protamin	20	20.7	0.04
Aktin mit Protamin	40	22.5	0.03
Aktin mit Protamin	80	24.0	0.03
L-Myosin mit Protamin	0	—	0.37
L-Myosin mit Protamin	10	2.8	0.36
L-Myosin mit Protamin	20	5.9	0.27
L-Myosin mit Protamin	40	11.1	0.14
L-Myosin mit Protamin	80	19.0	0.04
Aktin mit Polyäthensulfonat	0		0.36
Aktin mit Polyäthensulfonat	10		0.37
Aktin mit Polyäthensulfonat	20		0.36
Aktin mit Polyäthensulfonat	40		0.28
Aktin mit Polyäthensulfonat	80		0.19
L-Myosin mit Polyäthensulfonat	0		0.35
L-Myosin mit Polyäthensulfonat	10		0.07
L-Myosin mit Polyäthensulfonat	20		0.06
L-Myosin mit Polyäthensulfonat	40		0.04
L-Myosin mit Polyäthensulfonat	80		0.04

Die bevorzugte Bindung des Protamin an die Aktin-Komponente und des Polyäthensulfonats an die L-Myosin-Komponente kann auch auf folgende Weise bestätigt werden. Fig. 7a zeigt, dass die Zugabe steigender Mengen von reiner F-Aktin-Lösung die ATP-Spaltung durch ein Protamin-gehemmtes Aktomyosingel sehr steil bis zu dem normalen Wert ansteigen lässt, weil das Protamin vom Aktomyosin auf das Aktin übergeht. Dagegen beeinflusst der gleiche Aktin-Zusatz die ATP-Spaltung durch ein Polyäthensulfonat-gehemmtes Aktomyosingel fast garnicht, weil das

Polyäthensulfonat vom Aktin viel schlechter gebunden wird. Die Fig. 7b zeigt, dass der Zusatz von Flöckchen aus reinem L-Myosin gerade umgekehrt die ATP-Spaltung besser restituiert, die durch Polyäthensulfonat gehemmt ist, als die ATP-Spaltung in Anwesenheit von Protamin.

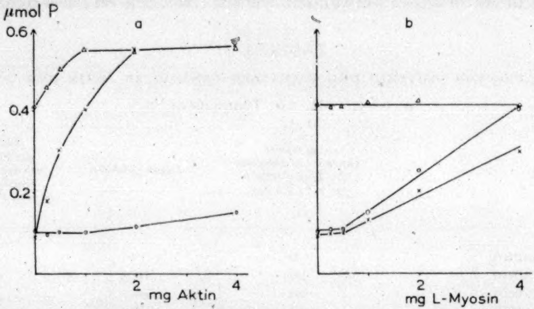

Fig. 7. Die Hemmung der Mg-aktivierten, natürlichen Aktomyosin ATPase durch Interaktions-Inhibitoren in Gegenwart von freiem Aktin und freiem L-Myosin (μ, 0.06; pH, 7.0; Temperatur, 25°). Ordinate: Spaltungsgeschwindigkeit in μmol P × min^{-1} × mg^{-1} des ursprünglichen Aktomyosin. Abszisse: (a) Milligramme an hinzugefügtem Aktin; (b) Milligramme an hinzugefügtem L-Myosin. (Die eigene ATP-Spaltung des L-Myosins wird in einem gesondertem Versuch bestimmt und ist von den dargestellten Aktomyosinspaltungskurven abgezogen). △—△, ATP-Spaltung in Abwesenheit von Interaktions-Inhibitoren; ×—×, ATP Spaltung in Anwesenheit von 20 Äquivalenten (siehe Fig. 1) von Protamin; ○—○, ATP Spaltung in Anwesenheit von 20 Äquivalenten (siehe Fig. 1) von Polyäthensulfonat.

Die Beobachtungen des Abschnitts V zeigen übereinstimmend, dass Protamin vorwiegend von dem stärker sauerem Aktin und Polyäthensulfonat vorwiegend von dem weniger sauerem L-Myosin gebunden wird.

ANHANG

Im Gegensatz zu den niedrig molekularen Interaktions-Inhibitoren (EDTA, Fuadin usw.) bleiben die hochpolymeren Inhibitoren Polyäthensulfonat und Protamin auch dann noch weitgehend an das Aktomyosin gebunden, wenn die Fibrillen oder Aktomyosin-Flocken in eine inhibitorfreie Lösung übertragen werden. Auch mehrmalige Erneuerung der inhibitorfreien Lösung ändert daran wenig.

Trotzdem ist diese Bindung nicht irreversibel. Denn Protamin-haltiges Aktomyosin gibt das Protamin vollständig ab und spaltet ATP wieder mit der normalen Rate von 0.33 μmol P × mg Eiweiss^{-1} × min^{-1}, wenn es sechsmal umgefällt ist. Dies kann darauf beruhen, dass das gebundene Protamin auf Grund einer Verschiebung des Bindungsgleichgewichtes durch die hohe Ionenstärke der Aktomyosinlösung abgegeben wird (vergl. KATCHALSKI *et al.*[4]). Ausserdem fällt in der Aktomyosinlösung der Diffusionsweg weg, der von dem abdissozierten Protamin in Fibrillen oder Flocken zurückgelegt werden muss. Es ist nicht untersucht, wie gross die Rolle des einen und des anderen Faktors ist. Die Mg-aktivierte ATP-Spaltung durch Polyäthensulfonat behandeltes Aktomyosin ist auch nach sechsfacher Umfällung noch sehr klein (0.05 μmol P × mg Eiweiss^{-1} × min^{-1}).

Vollständige Aufhebung sowohl der Protaminhemmung wie der Polyäthensulfonathemmung erfolgt dagegen selbst im Gelzustand des Aktomyosin ($\mu = 0.05$), wenn das Protamin behandelte Gel mit Polyäthensulfonat titriert wird und *vice versa* (siehe Literatur 4–10). In Fig. 8 ist Aktomyosingel je einmal mit 20 Äquivalenten und mit 40 Äquivalenten von Protamin und je einmal mit 20 Äquivalenten und mit 40 Äquivalenten von Polyäthensulfonat versetzt. Es ist gemessen wie sich die Spaltungsrate des ATP verändert, wenn die protaminhaltigen Aktomyosingele mit Polyäthensulfonat und polyäthensulfonathaltigen Aktomyosingele mit Protamin titriert werden. Die Abbildung zeigt, dass die anfänglich gehemmte ATP-Spaltung bis zum Äquivalentpunkt der beiden Polyelektrolyte enthemmt wird und im Überschuss des Titrationsmittels wieder abfällt (zur Identifizierung der einzelnen Titrationskurven vergleiche die Legende zur Fig. 8). Die elektrostatische Affinität der beiden Inhibitoren zueinander ist offenbar sehr viel grösser, als ihre Affinität zum Aktomyosin. Infolgedessen verbindet sich der eiweissgebundene Inhibitor mit dem Inhibitor der Titration. Im Äquivalenzpunkt ist der gesamte Inhibitor vom Aktomyosin auf den Inhibitor der Titration übergegangen. Dies geht daraus hervor, dass die Spaltungsrate des ATP in den Äquivalenzpunkten der vier Kurven die normale Höhe erreicht. Im Überschuss des Titrationsinhibitors wird die Spaltung natürlich wieder gehemmt.

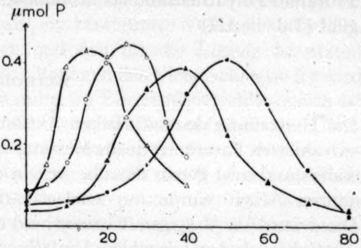

Fig. 8. Die Enthemmung der durch Polyelektrolyte gehemmten Mg-aktivierten natürlichen Aktomyosin ATPase durch einen Polyelektrolyten entgegengesetzter Ladung (μ, 0.05; pH, 7.0; Temperatur, 25°). Ordinate: Spaltungsgeschwindigkeit in μmol P × min^{-1} × mg^{-1}. Abszisse: Äquivalente (siehe Fig. 1) des enthemmenden Polyelektrolyten. Aktomyosingel gehemmt durch: △—△, 20 Äquivalenten an Protamin; ▲—▲, 40 Äquivalenten an Protamin; ○—○, 20 Äquivalenten an Polyäthensulfonat; ●—●, 40 Äquivalenten an Polyäthensulfonat.

Dass in diesen Titrationsversuchen der gebundene Inhibitor wirklich entfernt und nicht nur in seiner Wirkung neutralisiert wird, lässt sich in Systemen niedriger Ionenstärke nur für F-Aktin-Lösungen zeigen. Wird zu solchen Lösungen Protamin

TABELLE III

DIE ABTRENNUNG DES PROTAMIN AUS SEINER BINDUNG AN AKTIN DURCH BILDUNG EINES PROTAMIN-POLYÄTHENSULFONAT KOMPLEXES

μ, 0.05; pH, 7.0; Temperatur, 25°.

Zustand des Aktin	*Eiweiss*	*Argeningehalt*
Ursprüngliche F-Aktin Lösung	~ 5 mg/ml	43 Mol/10^5 g Eiweiss
Überstand nach Aktinfällung durch Protamin	Spuren (Trichloressigsäure-Fällung)	
Waschwasser der 3 Waschungen Protamin-Aktinat	Spuren	
Verwandlung des Protamin-Aktinat in Protamin-Polyäthensulfonat: Überstand	3.9 mg/ml	45 Mol/10^5 g Eiweiss

hinzugegeben, so bildet sich ein unlöslicher Aktin-Protamin Komplex. Wenn dieser Aktin-Protamin Komplex von dem protaminhaltigen Überschuss abgetrennt und in einer Polyäthensulfonatlösung mit äquivalentem Polyäthensulfonatgehalt suspendiert wird, so verwandelt sich der Aktin-Protamin Komplex in einen unlöslichen Protamin-Polyäthensulfonat Komplex, während das freie Aktin in den Überstand geht (Tabelle III).

METHODISCHER TEIL

Die Herstellung des natürlichen Aktomyosins, L-Myosins, Aktins und der Glyzerin extrahierten Fasern, sowie die Messung der Spannungsentwicklung und des Dehnungswiderstandes ist genau dieselbe, wie in der voranstehenden Arbeit[1]. Während dieser ganzen Arbeit wurde nur eine natürliche Aktomyosin-Stammlösung verwendet. Diese wurde in 50 %igem Glyzerin und 0.3 *M* KCl in der Kühltruhe aufbewahrt und vor Gebrauch durch 2 malige Umfällung vom Glyzerin befreit.

Die Bestimmung der ATPase Aktivität erfolgt im Gelzustand mit 0.025 %, im Solzustand (und im Falle des L-Myosingels mit Mg) mit 0.05 % Eiweiss, in Histidin-Puffer pH 7.0. Vor Zugabe des ATP werden die Ansätze 5 min lang mit den Inhibitoren bei 25° magnetisch gerührt. Bei der Enthemmung der Polyelektrolytwirkung an das Aktomyosingel (vergl. Figuren 7 und 8) werden die Ansätze nach Zugabe des enthemmenden Mittels noch zusätzlich 5 min gerührt. Die Spaltungszeit dauert 3–10 min. Es wird in Betracht gezogen, dass während dieser Zeit nicht mehr als 25 % des gesamt ATP-s gespalten werden soll. Die angegebenen Spaltungsraten werden durch Doppelbestimmung festgelegt. Das freigelegte Phosphat wird nach ROCKSTEIN UND HERRON[11] bestimmt.

Eiweiss wurde mit der Biuretmethode von GORNALL *et al.*[12] bestimmt; die Extinction wurde bei 320 mμ gemessen[13]. Wenn die Konzentration von Muskelproteinen in Anwesenheit von Protamin bestimmt werden muss (vergl. Bestimmung der Protaminbindung) wurde die Eiweisskonzentration zusätzlich auch noch aus der Lichtabsorption bei 280 mμ berechnet, weil Protamin bei 280 mμ nicht absorbiert. Eine kleine zusätzliche scheinbare Absorption durch "light scattering" wurde nach FRAENKEL-CONRAT[14] durch Absorptionsbestimmung bei 360 und 320 mμ sowie geradlinige Extrapolation der Werte auf 280 mμ zu eliminieren versucht.

Zur Bestimmung der Bindung von Protamin und Polyanetholsulfonat wurden Proben von Aktomyosin, L-Myosin und F-Aktin mit verschiedenen Konzentrationen von Protamin und Polyanetholsulfonat unter genau den gleichen Bedingungen inkubiert, unter denen die Proben für die Bestimmung der ATP-Spaltung inkubiert wurden. Doch wurde die Bindung immer in Abwesenheit von ATP bestimmt. Aktomyosin und L-Myosin wurden dann mit 17,900 × *g*, das F-Aktin mit 144,700 × *g*, abzentrifugiert. Kontrollen ergaben, dass Protamin und Polyanetholsulfonat in der gleichen Zeit auch mit 144,700 × *g* nicht am Boden der Zentrifugenzelle angereichert werden.

Die gebundene Menge an Polyanetholsulfonat ergibt sich aus der Differenz der Polyanetholsulfonat-Konzentration vor und nach Abzentrifugieren des Eiweisses. Die Konzentration vor dem Abzentrifugieren ist durch die Zusammensetzung der Versuchslösung bekannt. Die Konzentration nach dem Abzentrifugieren ergibt sich aus der Lichtabsorption des Polyanetholsulfonats im Überstand bei der Wellenlänge

284 mμ. Der Extinktionskoeffizient bezogen auf die normale Lösung beträgt $1.85 \cdot 10^3$ bei pH 7.0. Um den Konzentrationsunterschied möglichst gross zu machen werden möglichst konzentrierte Aktomyosin-Lösungen verwendet.

Die gebundene Menge an Protamin wird im Niederschlag bestimmt. Hierfür werden die Niederschläge mit einer abgemessenen Menge von 0.1 *N* NaOH gelöst und mit einer abgemessenen Wassermenge auf ein bestimmtes Volumen aufgefüllt. Aus der Differenz zwischen diesen Volumen und den für die Lösung benutzten Volumen von NaOH und Wasser ergibt sich das Volumen des Niederschlages. Es wird angenommen, dass die Menge des freien Protamins im Eiweissniederschlag gleich ist dem Niederschlagvolumen multipliziert mit der Protamin-Konzentration des Überstandes. Der Protamingehalt im Überstand und Niederschlag wurde mit der Arginin-Reaktion von SAKAGUCHI[15], nach der Modifizierung von KEYSER[16] gemessen. (Laut unseren Erfahrungen ist die Empfindlichkeit der Reaktion grösser mit 0.25 *N* Natriumhypochlorit und 2 min Wartezeit vor Zugabe der Harnstofflösung). Von dem Gesamtarginin des Niederschlags wurde das Arginin des eingeschlossenen, freien Protamin sowie das Arginin abgezogenen, das als Baustein in dem untersuchten Eiweiss vorhanden ist. Dieses Baustein-Arginin wurde in einer Protamin freien Kontrolle des fraglichen Eiweisses mit gleicher Methode bestimmt.

Das Protaminsulfat erhalten wir von der Firma L. Light et Co.; sein Sulfatgehalt beträgt 18.9%, sein Arginingehalt 69%; es enthält kein im u.v.absorbierendes Material. Für das *Polyäthensulfosaures Natrium* danken wir der Firma Hoechst; der Sulfatgehalt ist 21.4%. Für das *Polylysinhydrobromid* bedanken wir uns bei Dr. A. BERGER, Weizmann Institute of Science, Rehovoth, Israel, und für das *Cetyltrimethylammoniumchlorid* und *Dodecylsulfat* bei Dr. H. HOFFMANN-BERLING im hiesigen Institut. *Heparin* erhalten wir von S. A. F. Hoffmann-La Roche und Co., Schweiz, und *Polyanetholsulfosaures Natrium* (Liquoid) von der Deutschen Hoffmann-La Roche A.G., Grenzach. *Germanin* ist ein Bayer-Präparat. *ATP* liefert uns die Firma Pabst. Alle anderen Materialien sind handelsübliche *p.a.* Präparate.

DANK

Herrn Professor H. H. WEBER danken wir für hilfreiche Diskussionen, Fräulein R. MÜLLER und Frau E. ARNOLD für die gewissenhafte und fleissige Unterstützung bei der Durchführung dieser Arbeit.

ZUSAMMENFASSUNG

1. Es wird gezeigt, dass es unter den hochmolekularen Elektrolyten sowohl Interaktions-Inhibitoren als auch Myosin-ATPase-Gifte gibt.

2. Es wird gezeigt, dass Ca-aktiviertes Aktomyosin im Gelzustand und im Solzustand ATP mit quantitativ derselben Geschwindigkeit spaltet, mit der reines L-Myosin unter den gleichen Bedingungen ATP spaltet, falls die Spaltung durch Aktomyosin auf die Gewichtseinheit der im Aktomyosin enthaltenen L-Myosin-Komponente bezogen wird.

3. Ebenso ist die Rate der Ca-aktivierten ATP-Spaltung identisch in Aktomyosin und L-Myosin Systemen, wenn Myosin-ATPase-Gifte oder Interaktions-Inhibitoren anwesend sind. Unter Myosin-ATPase-Giften fällt die Spaltung in beiden Systemen

in gleichem Umfang ab; unter Interaktions-Inhibitoren wird die Spaltung in beiden Systemen überhaupt nicht beeinflusst.

Die Beobachtungen unter 2 und 3 führen zu dem Schluss, dass die Ca-aktivierte ATP-Spaltung immer—auch in Aktomyosingelen—auf der Wirkung der L-Myosin-ATPase beruht. Nur die Anwesenheit von Mg bewirkt in Aktomyosingelen eine enzymatische Interaktion zwischen Aktin und Myosin.

4. Die Interaktionshemmung durch Protamin und Polyäthensulfonat beruht nicht auf einer Erhöhung der Ionenstärke und ist unabhängig von der Eiweissladung im pH-Bereich von 6–9.

5. Die Interaktionshemmungen durch das Polyanion Polyanetholsulfonat und durch das Polykation Protamin werden für alle Konzentrationen der Polyionen gleich, wenn die Hemmung nicht auf die zugegebene, sondern auf die durch Aktomyosin gebundene Menge der Polyionen bezogen wird.

6. Das Polykation Protamin wird überwiegend vom F-Aktin, das Polyanion Polyäthensulfonat fast ausschliesslich vom L-Myosin gebunden.

7. Die Verbindung von hochpolymeren Interaktions-Inhibitoren mit den kontraktilen Proteinen dissoziiert langsamer und unvollständiger als die Verbindung zwischen niedrig molekularen Inhibitoren und den Proteinen. Es wird gezeigt, dass auch die Bindung der Polyelektrolyte an das Aktomyosin reversibel ist.

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PURIFICATION AND PROPERTIES OF MALATE SYNTHETASE

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SUMMARY

Procedures are described for the purification of malate synthetase from baker's yeast and from glycollate-grown *Pseudomonas ovalis* Chester. The properties of the enzymes are closely similar: both are optimally active at pH 8.5, but that from yeast shows greater activity below this pH than that from *Ps. ovalis*.

The K_m for glyoxylate was $9.3 \cdot 10^{-5}\,M$ (yeast) and $6.3 \cdot 10^{-5}\,M$ (*Pseudomonas*). The K_m for acetyl–S–CoA was $< 1 \cdot 10^{-5}\,M$.

The enzymes require Mg^{++} for activity ($K_m = 5 \cdot 10^{-4}\,M$) and are competitively inhibited by the C_2-acids oxalate ($K_i = 1.9 \cdot 10^{-5}\,M$) fluoroacetate ($K_i = 2.46 \cdot 10^{-4}\,M$) and glycollate ($K_i = 3.08 \cdot 10^{-4}\,M$). The purified enzymes are specific for glyoxylate, and do not catalyse the cleavage of acetyl–S–CoA in the presence of oxaloacetate, pyruvate, α-ketoglutarate, glyoxal, glycolaldehyde, formaldehyde, or acetaldehyde.

In the presence of glyoxylate, there was no reaction with S,N-diacetyl β-mercaptoethylamine, S-acetyl pantetheine, propionyl coenzyme A or butyryl coenzyme A, but fluoroacetyl coenzyme A was split at approximately one quarter of the rate observed with similar concentrations of acetyl–S–CoA. Malyl–S–CoA was not split by the enzymes, either alone or in the presence of glyoxylate. The equilibrium of the reaction strongly favours malate formation: no reversal of the reaction was detected.

Procedures are described for the preparation of [^{35}S]coenzyme A, which was used to test for the possible formation of an acyl enzyme as an intermediate in the malate synthetase reaction. The non-enzymic isotopic exchange of ^{35}S from CoA^{35}SH with acetyl–S–Coenzyme A observed was not affected by the presence of malate synthetase. The properties of malate synthetase are compared with those of the citrate-forming condensing enzyme.

INTRODUCTION

Malate synthetase, which catalyses the reaction

$$\text{acetyl}\cdot\text{S}\cdot\text{CoA} + \text{glyoxylate} + H_2O \longrightarrow \text{malate} + \text{CoA}\cdot\text{SH} \qquad (1)$$

The following abbreviations are used: CoA and CoA·SH = coenzyme A; R·S·CoA = acyl coenzyme A; DPNH = reduced diphosphopyridine nucleotide; Tris = tris(hydroxymethyl)aminomethane; ATP = adenosine triphosphate.

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was discovered in extracts of acetate-grown *Escherichia coli* by WONG AND AJL[1], who reported a 50-fold purification from such extracts. Like other enzymes catalysing the interaction of the methyl group of acetyl–S–CoA with keto groups of α-ketonic acids (*e.g.* the citrate-forming condensing enzyme[2]), malate synthetase catalyses the *de novo* formation of carbon-carbon bonds, and hence plays an important role in the biosynthesis of cell constituents. Although the enzyme appears to be absent from mammalian cells[3], it has been shown to be of importance in the microbial synthesis of cellular materials from C_2-compounds such as acetate[4,5], glycollate[6], glycine[7] and oxalate[8], and to be a key enzyme in the net conversion of fat to carbohydrate in fatty seedlings[9,10]. Whilst the discoverers of malate synthetase found the enzyme to be present only in extracts of acetate-grown micro-organisms[11], the development of direct and more sensitive methods of assay[12] have shown malate synthetase to be widely distributed: it has been found in yeast[13], protozoa[14,15] algae[16], fungi[17] and many bacteria under widely different conditions of growth[18,19].

In view of the importance of the enzyme in intermediary metabolism, it was of interest to study the properties of malate synthetase in a purified form. Convenient and reproducible procedures are reported for the purification of the enzyme from two sources: baker's yeast, which, though not particularly rich in malate synthetase, is readily available in bulk, and glycollate-grown *Pseudomonas ovalis* Chester, crude extracts of which contain malate synthetase of relatively high specific activity. The enzymes isolated from both sources exhibit closely similar properties, but differ in their affinity for ion-exchange cellulose resins, and hence probably in their charge.

MATERIALS AND METHODS

Materials

Coenzyme A, sodium glyoxylate and tris(hydroxymethyl)aminomethane (Tris) were obtained from the Sigma Chemical Corp., St. Louis, Mo., U.S.A. Other materials were obtained as indicated in the text.

Preparation of substrates

Acyl–S–CoA derivatives: Acetyl–S–CoA was prepared according to STADTMAN[20] and purified by chromatography in 50% ethanol–0.1 *M* potassium acetate, pH 4.5. The material eluted from the chromatogram was stable in the frozen state for many weeks. Propionyl–S–CoA and butyryl–S–CoA were prepared from the corresponding anhydrides under the same conditions. Fluoroacetyl–S–CoA was prepared by the mixed anhydride method of BRADY[21], using sodium fluoroacetate (Monsanto, 90% pure) as starting material, the free fluoroacetic acid being generated by the addition of one equivalent of anhydrous sulphuric acid to one equivalent of sodium fluoroacetate in sodium-dried ether. As described by BRADY[21], the fluoroacetyl–S–CoA was extremely labile in alkaline solution.

Malic anhydride was prepared by the reaction of thionyl chloride with di silver L-malate by the method of DENHAM AND WOODHOUSE[22] and assayed 96% pure by the hydroxamic acid test. It was reacted with CoA–SH under the usual conditions[20] (0.2 *M* potassium bicarbonate solution at 0°). A thiolester was obtained in good yield, and possessed similar properties to acetyl–S–CoA, being nitroprusside negative before alkaline hydrolysis and positive after treatment with 2% methanolic sodium hydroxide[20]. It was not known which carboxyl of malate was esterified.

Other thiolesters: S,N-diacetyl-β-mercaptoethylamine was prepared by the acetylation of β-mercaptoethylamine ("Labaz" Bécaptan, 10% adjusted to pH 7.5 with *N* HCl) with acetic anhydride in 0.2 *M* potassium bicarbonate solution at 0°[20]. S-acetyl pantetheine was prepared by the reduction of pantethine (Nutritional Biochemicals Corp.), with solid sodium borohydride. The appearance of free sulfhydryl was followed quantitatively by the nitroprusside technique of GRUNERT AND PHILLIPS[23] and when reaction was complete a sample was transferred to 0.2 *M* potassium bicarbonate and acetylated.

Isotopic compounds

Isotopic materials were purchased from the Radiochemical Centre, Amersham, Bucks (England).

[1-^{14}C]acetyl CoA was prepared from [1-^{14}C]acetic anhydride by the method of STADTMAN[20] and was purified as described above.

Sodium [1-^{14}C]glyoxylate was prepared by treatment of Ca[1-^{14}C]glyoxylate with Dowex 50 resin in the Na^+ form.

[1-^{14}C]malate was prepared enzymically by the action of purified yeast malate synthetase upon acetyl–S–CoA and [1-^{14}C]glyoxylate. A reaction mixture (final volume 2.0 ml) contained 3 μmoles of acetyl–S–CoA, 3.75 μmoles of [1-^{14}C]glyoxylate, 40 μmoles of Tris·HCl pH 7.2, 20 μmoles of $MgCl_2$ and 2.5 Units of yeast malate synthetase. The mixture was incubated for 30 min at room temperature. The reaction mixture was then streaked onto the "origin" line of a sheet of Whatman No. 3 paper and developed by descending chromatography in butanol–propionic acid–water (100:50:70)[24] overnight. There was a strong radioactive band, the position of which corresponded to that of a marker of L-malate, which was eluted. Its identity was confirmed by co-chromatography of a sample with L-malic acid in the two-dimensional solvent system of KORNBERG[25]. The yield of [1-^{14}C]malate was 2.82 μmoles (determined chemically by the method of HUMMEL[26]) with a specific radioactivity of $1.85 \cdot 10^6$ counts/min/μmole (counted in a gas flow counter at infinite thinness).

[^{35}S]CoA was prepared biosynthetically by the growth of *Escherichia coli* strain *w* (ATCC 9637) in the presence of [^{35}S]sulphate (carrier free, in aqueous solution) and a relatively sulphur-poor medium in which acetate was the sole source of carbon. In 9 l the medium contained 57.4 g of disodium hydrogen phosphate·$2H_2O$, 16.8 g of potassium dihydrogen phosphate, 73.8 g of sodium acetate, 48.15 g of ammonium chloride·$4H_2O$, 0.72 g of magnesium chloride·$6H_2O$, and 0.036 g of ferrous sulphate·$6H_2O$. Approximately 2 mC of [^{35}S]sulphate were added to the culture medium just before inoculation with 400 ml of a culture of *E. coli* grown in a similar medium in a shake flask overnight. The culture was allowed to grow for 24 h at 30° with vigorous aeration by sterile filtered air. Growth was followed by nephelometric measurements, and uptake of ^{35}S by counting portions of the supernatant solutions after centrifugation of a portion of the culture. The yield of cells was 0.18 mg dry weight/ml of culture and the uptake of [^{35}S] was almost quantitative. The cells were collected by centrifugation in 1 l polythene bottles and were washed once with water and resuspended in a small volume of 0.1 *M* Tris·HCl, pH 7.0. The slurry was poured into a HUGHES press[27] at −30° and crushed when the press had reached −25°. The crushed material was resuspended in 0.1 *M* Tris·HCl, pH 7, producing an extremely viscous suspension; it was rapidly liquefied by addition of a few crystals of deoxy-

ribonuclease (L. Light and Co., Colnbrook, Bucks.). The suspension was then centrifuged at 20,000 × g at 0° and the supernatant solution deproteinized by heating in boiling water for 5 min. The coagulated protein was removed by centrifugation and the supernatant solution was assayed enzymically for CoA by the disappearance of acetyl phosphate in the presence of arsenate[28], using an *E. coli* crush as a source of transacetylase. It was necessary to free the enzyme preparation of endogenous CoA–SH by treatment with IRA-400 resin in the chloride form and to carry out the assay in the presence of 0.1 M potassium fluoride to inhibit a powerful acetyl phosphatase present in the extract. The boiled supernatant, containing approximately 0.03 mg CoA–SH/ml, was applied to a sheet of Whatman No. 3 paper as a narrow band and subjected to ionophoresis in pyridine–acetic acid buffer, pH 3.6, in a MICHL[29] toluene-cooled apparatus. Radioautographs of the dried sheet showed a radioactive band in the position of the marker CoA; this band was eluted and purified by co-chromatography with authentic CoA in 50 % ethanol–0.1 M potassium acetate buffer, pH 4.5[20]. After purification of [^{35}S]CoA it was found that the product was largely in the disulphide form. It was reduced to the sulfhydryl form by treatment with sodium borohydride (solid) followed by acidification to pH 3.

Purification of malate synthetase from baker's yeast

Step 1. Preparation of sonic extract: All operations were carried out at 2°. Fresh baker's yeast (400 g) was mixed with 800 ml of buffer, containing 0.005 M Tris·HCl, pH 8.0 and 0.001 M $MgCl_2$ (hereafter designated "Mg–Tris buffer"), and subjected for 20 min to the output of a 500 W Mullard magnetostrictor oscillator, operating at 19 kc/sec. The suspension was centrifuged at 14,000 × g for 30 min, and the slightly turbid yellow supernatant solution was filtered through glass wool to remove particles of fatty material.

Step 2. Ammonium sulphate precipitation and dialysis: To the supernatant solution was added, with mechanical stirring, ammonium sulphate to 75 % saturation (516 g/l). The precipitated material was separated by centrifugation, dissolved in the minimum volume of Mg–Tris buffer and dialysed overnight against Mg–Tris buffer. The slight precipitate was removed by centrifugation.

Step 3. First column chromatography: Approx. 20 g of diethylaminoethyl cellulose (DEAE-cellulose, Whatman DE 50) were suspended in 600 ml Mg–Tris buffer and the pH was adjusted to 8.0 by the addition of 4 N HCl. Of the resultant slurry, approx. one third was poured into a chromatographic column (2 cm × 30 cm), plugged at its lower end with glass wool. (It is important not to use a sintered disc for supporting the DEAE-cellulose, as this rapidly clogs.) The column was equilibrated overnight by running through it 1–2 l of Mg–Tris buffer.

The dialysed yeast fraction was now applied to the column and was eluted with Mg–Tris buffer. At this stage, the malate synthetase did not adhere to the column and was quantitatively eluted in the hold-up volume. Effluent from the column was collected with an automatic fraction collector.

Step 4. Ammonium sulphate precipitation and dialysis: The fractions containing the enzyme were pooled and the protein was precipitated by the addition of ammonium sulphate to 75 % saturation. The precipitated material was collected by centrifugation, dissolved in the minimum volume of Mg–Tris buffer and was dialysed overnight against Mg–Tris buffer.

Step 5. Second column chromatography: The dialysed material was applied to a DEAE-cellulose column, prepared and equilibrated as described under *Step 3*, and 40–50 ml of Mg–Tris buffer were run through the column. At this stage, the malate synthetase adhered to the column. A linear gradient of $MgCl_2$ was now applied to the column. This was obtained by allowing 400 ml of 0.005 *M* Tris·HCl buffer, pH 8.0 containing 0.050 *M* $MgCl_2$, to mix, with constant stirring, with 400 ml of Mg–Tris buffer and running the resultant mixture continuously through the column. Effluent fractions were collected automatically. (It is convenient to collect fractions of approx. 7 ml, and to maintain the flow-rate of eluent at 40–45 ml/h.) Malate synthetase was eluted from the column as a narrow peak with the maximum activity appearing at a chloride concentration of 0.02 *M* (Fig. 1).

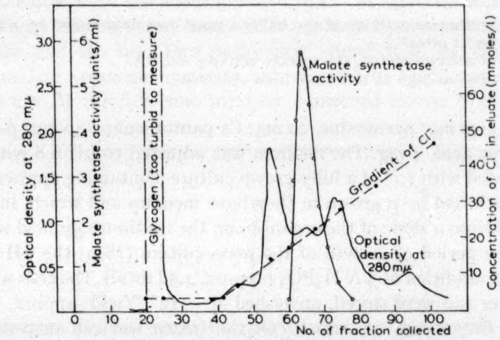

Fig. 1. Elution of the malate synthetase of baker's yeast from DEAE-cellulose. For conditions, see text. The continuous line represents the O.D. of fractions (vol. = 7 ml) at 280 mμ (1 cm light path); ○—○, malate synthetase activity; ●—●, gradient of chloride concentration.

Step 6. Alumina C_γ gel adsorption: The fractions containing malate synthetase at specific activities greater than 3 were pooled, and C_γ gel suspension (0.2 vol; 27.5 mg/ml) was added. The enzyme was adsorbed on the gel, which was separated by brief centrifugation: it was eluted by treatment of the gel with a mixture of 0.05 *M* potassium phosphate buffer, pH 7.82 and 0.01 *M* $MgCl_2$. It has been found repeatedly with the malate synthetase of both baker's yeast and *Pseudomonas ovalis* Chester (see later) that serious inactivation occurs if enzyme of this or higher purity is fractionated with ammonium sulphate. However, the enzyme eluted from the C_γ gel represents a purification of 200–300 fold from crude baker's yeast, was free of all contaminating enzymes for which tests were made and was stable at 2° for at least a month. The purification procedure is summarized in Table I.

Purification of malate synthetase from Pseudomonas ovalis *Chester*

Growth of the organism: Mass cultures of the organism were obtained through the kindness of Dr. D. E. HERBERT and Mr. R. ELSWORTH from the Ministry of Supply, Microbiological Research Establishment, Porton, nr. Salisbury, Wilts. (England). Batches of the organism (NCIB 8296; ATCC 8209) were grown, with vigorous aeration, in 100 l of medium containing per litre: $(NH_4)_2SO_4$, 12 g; $NaH_2PO_4 \cdot H_2O$, 3 g; K_2SO_4, 3.5 g; sodium glycollate, 10 g; $MgSO_4$, 4 mmoles; $CaCl_2$, 0.4 mmoles;

TABLE I

SUMMARY OF PURIFICATION PROCEDURE OF MALATE SYNTHETASE FROM BAKER'S YEAST

Step	Volume of solutions (ml)	Units	Protein (mg)	Specific activity (Units/mg protein)	Yield
Baker's yeast	—	—	—	0.036*	—
1. Sonic extract of yeast	705	403	3370	0.12	100
2. Dialysed ammonium sulphate precipitate	110	330	2200	0.15	82
3. Eluate from first column chromatogram	94	280	451	0.62	69
4. Dialysed ammonium sulphate precipitate	10.5	266	336	0.79	66
5. Pooled selected fractions from second column chromatogram	50	182	39	4.67**	45
6. Eluate from C_γ-gel adsorption	5	134	16.7	8.0***	33

* The malate synthetase activity of the baker's yeast was determined on a sample of yeast crushed in the HUGHES press[27].

** At the peak of activity eluted, the specific activity was 6.5.

*** Specific activities as high as 12.1 have been obtained in this step.

$Fe(NH_4)_2(SO_4)_3$, 40 mg; pyridoxine, 20 mg; Ca pantothenate, 40 mg; *p*-aminobenzoic acid, 20 mg; folic acid, 4 mg. The medium was adjusted to pH 6.8 with 3 *N* H_3PO_4 and was inoculated with 10 l of a fully-grown culture (containing approx. 10 g dry wt. of bacteria) which had been grown in the above medium and which, in its turn, had been inoculated from a slope of the organism on the medium solidified with 2 % (w/v) agar. During the period of growth of the mass culture (18 h) the pH was adjusted to 6.8 by periodic addition of 3 *N* H_3PO_4 (approx. 1.5 l total). The cells were harvested by centrifugation and were stored, unwashed at —12°. Yield: approx. 500 g wet wt.

Steps 1–3, Preparation of extracts: Of the frozen wet cell suspension, 70–80 g were thawed and subjected for 5 min to the output at 25 kc/sec of a 600 W Mullard magnetostrictor oscillator. The resultant viscous suspension was rapidly liquefied by addition of 1 crystal of deoxyribonuclease and was centrifuged for 15 min at 20,000 × *g*, 0°, in an International refrigerated centrifuge. The precipitated material was suspended in its own volume of Mg–Tris buffer and was subjected to a further treatment for 5 min in the magnetostrictor oscillator. After centrifugation for 15 min at 20,000 × *g*, 0°, the precipitated material was discarded, and the supernatant solutions obtained from both treatments in the magnetostrictor oscillator were combined. These were dialysed overnight against twice 2 l of Mg–Tris buffer at 2°.

Step 4. Column chromatography: After dialysis, the bacterial extract was centrifuged for 30 min at 0°, 20,000 × *g* and the intensely pigmented supernatant solution (vol. approx. 80 ml) was applied to the column. Mg–Tris buffer (approx. 90 ml) was run through the column at approx. 40 ml/h until the effluent was clear and contained no material which absorbed light at either 280 or 260 mμ. (The highly pigmented material does not adhere to the column, and, unlike the similar fraction obtained from baker's yeast, is free of malate synthetase activity. It contains over half the total protein of the extract.) Malate synthetase was eluted from the column by a linear gradient of $MgCl_2$ as described above, but using 0.1 *M* $MgCl_2$ instead of 0.05 *M*-$MgCl_2$ in the "strong buffer" vessel. The peak of enzymic activity corresponded to a chloride concentration in the eluate of 0.06 *M*. The bulk of the enzyme was eluted in approx. 77 ml, the peak of specific activity was 7.1.

Step 5. Concentration of the eluate: The fractions eluted from the column which contain malate synthetase activity at greater than 1.5 Units/mg of protein were pooled and mixed with 0.05 vol. of 2 *M* $MgCl_2$. Solid ammonium sulphate was added, with stirring, to final concentration of 75 % saturation (516 g/l) and the precipitated protein, collected by centrifugation for 5 min at 20,000 × *g*, 0°, was suspended in 10 ml of Mg–Tris buffer. This bright yellow suspension was dialysed against Mg–Tris buffer overnight, during which time the remainder of the undissolved protein passed into solution and most of the yellow colour was lost. The resultant solution of malate synthetase was still contaminated with condensing enzyme and was purified further by a second column chromatography.

Step 6. *Second column chromatography:* The dialysed material (vol. 25 ml) was applied to a DEAE-cellulose column equilibrated with Mg–Tris buffer, washed with the buffer, and eluted with the linear gradient of Cl^- in Mg–Tris buffer as described above. The effluent fractions were analysed for protein by measurement of the light absorbed at 280 and 260 mμ. Two peaks were eluted from the column; the first contained no malate synthetase activity but was rich in condensing enzyme whilst the second, at 0.06 *M* chloride concentration, contained malate synthetase (Fig. 2).

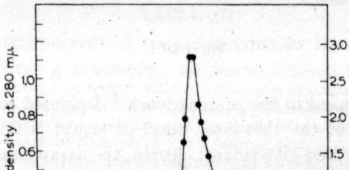

Fig. 2. Elution of the malate synthetase of *Ps. ovalis* Chester from DEAE-cellulose. For conditions, see text. The continuous line represents the O.D. of fractions (vol. 7 ml) at 280 mμ (1 cm light path); ●—●, malate synthetase activity.

All fractions of this peak containing malate synthetase at specific activities greater than 4 were combined and a sample of this pooled material (total vol. 45 ml, specific activity 6.9) was used for the studies of properties of the enzyme. The purification procedure is summarized in Table II.

Definition of unit and specific activity

One unit of malate synthetase was defined as the amount which catalyses the cleavage of 1 μmole of acetyl–S–CoA/min under the conditions of assay used[12].

Specific activity was expressed as Units/mg of protein.

Estimation of chloride

The chloride concentration of the $MgCl_2$ gradients was determined by the spectrophotometric method of WEST *et al.*[30].

TABLE II

SUMMARY OF PURIFICATION PROCEDURE OF MALATE SYNTHETASE FROM *Ps. ovalis* CHESTER

For experimental details, see METHODS.

Step	*Vol. (ml)*	*Units/ml*	*Protein concn. (mg/ml)*	*Specific activity*	*Total units*	*Yield (%)*
1. Crude sonic extract	141	17.6	117	0.15	1650	100
2. Combined supernatant solutions after centrifugation	82	19.2	51	0.37	1570	95
3. Combined supernatant solutions after dialysis and centrifugation	82	14.0	41	0.34	1150	70
4. Pooled selected fractions from 1st column chromatography	75	4.9	1.4	3.5*	367	22.2
5. Pooled selected fractions after precipitation with ammonium sulphate and dialysis	25	8.2	1.8	4.6	205	12.4
6. Pooled selected fractions after 2nd column chromatography	45	2.15	0.31	6.9**	96.6	5.86

* The peak of activity was 7.1.
** The peak of activity was 8.2.

RESULTS

Assay used

The enzymic assay used in the present work[12] depended on direct measurement of the rate of cleavage of the thio-ester bond of acetyl–S–CoA in the presence of malate synthetase, Mg^{++} and glyoxylate: clearly, the assay cannot be used if acetyl–S–CoA is removed other than by the action of malate synthetase. In preliminary experiments, it was found that acetyl–S–CoA was rapidly split by crude extracts of many micro-organisms, in the absence of added glyoxylate, if such extracts were suspended in phosphate buffer. This was found to be due to the presence of phosphotransacetylase[28], which catalyses the reaction (2)

$$\text{acetyl–S–CoA} + \text{phosphate} \longrightarrow \text{acetyl phosphate} + \text{CoASH} \tag{2}$$

In addition, the inhibition of the rate of enzymic cleavage of acetyl–S–CoA by excessive concentrations of acetyl–S–CoA proved to be most pronounced if phosphate buffers were used (see Table IV). These factors led to the adoption of Tris buffer as reaction milieu.

Removal of acetyl-S–CoA in the absence of glyoxylate can also be effected by the action of acetyl–S–CoA deacylases[31]. In the two organisms studied, the action of such enzymes appeared to be quantitatively insignificant: the O.D. at 232 mμ was constant even when crude extracts of yeast or *Pseudomonas ovalis* Chester were incubated with acetyl–S–CoA. Addition of glyoxylate to such systems led to rapid decrease of O.D., the initial rate of which was constant for the cleavage of at least 40 % of the total acetyl-S-CoA present. This initial rate was stoichiometrically related to the quantities of enzyme added (Fig. 3). Experiments with [1-^{14}C]acetyl–S–CoA and with [1-^{14}C]glyoxylate (Table III) showed that malate was the only labelled product of the reaction, and that the quantities of labelled malate formed were equivalent to the amounts of acetyl–S–CoA and of glyoxylate utilized. This was confirmed by chemical estimation of malate. No reaction was detected in the absence of acetyl-S-CoA, glyoxylate or malate synthetase, or in the complete system if the enzyme was boiled prior to incubation.

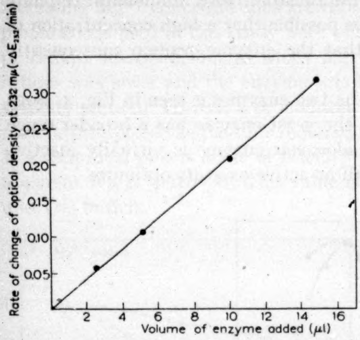

Fig. 3. Effect of malate synthetase concentration on the initial rate of cleavage of acetyl coenzyme A in the presence of glyoxylate. The reaction system contained, in 0.42 ml, 38 μmoles of Tris·HCl, pH 8.0; 2.5 μmoles of $MgCl_2$; 0.04 μmoles of acetyl-S-CoA and varying amounts of malate synthetase from *Ps. ovalis* (0.021 Units/μl). Sodium glyoxylate (0.2 μmoles) was added at zero time and the E_{232} recorded.

TABLE III

STOICHIOMETRY OF MALATE SYNTHETASE REACTION

The reaction mixture is Expt. A, containing 0.246 μmoles of acetyl-S-CoA, 10 μmoles of $MgCl_2$, 275 μmoles of potassium phosphate buffer, pH 8.0, 1.0 μmoles of sodium glyoxylate and 0.07 Units of malate synthetase, was incubated for 11 min at 25°. Malate formed was estimated by the method of HUMMEL[26]. The reaction mixture used in Expt. B contained 0.048 μmoles of [1-^{14}C]-acetyl-S-CoA, 37 μmoles of Tris·HCl, pH 8.0, 1 μmole of $MgCl_2$, 0.2 μmoles of glyoxylate and 0.02 Units of malate synthetase in a final volume of 0.42 ml. The ΔE_{232} was recorded until zero (8 min) and the contents of the cuvette were mixed with 0.8 ml of 90% ethanol. The reaction mixture used in Expts. C and D, containing 2 μmoles of $MgCl_2$, 54 μmoles of Tris·HCl buffer, pH 8.0, 1.07 Units of malate synthetase, 0.01 ml of [1-^{14}C]acetyl-S-CoA (Expt. C) or 0.14 μmoles of acetyl-S-CoA (Expt. D) and 0.2 μmoles of sodium glyoxylate (Expt. C) or 0.2 μmoles of [1-^{14}C]glyoxylate (Expt. D), were incubated for 15 min at 25°. The reaction was stopped by addition of 1 ml of 95% ethanol. The solutions were evaporated to dryness under N_2 at 60° and the radioactive constituents were analysed by chromatography and radioautography[15].

Expt. No.	*Source of enzyme*	*Decrease in Acetyl-S-CoA*		*Decrease in [1-^{14}C]glyoxylate*	*Malate formed*		*Radioactivity of products — other than malate*
		(μmoles)	*(counts/min)*	*(counts/min)*	*(μmoles)*	*(counts/min)*	*(counts/min)*
A	Baker's yeast	0.246	—	—	0.273	—	—
B	Baker's yeast	—	8412	—	—	8134	0
C	Pseudomonas ovalis	—	3700	—	—	3590	0
D	Pseudomonas ovalis	—	—	6810	—	6734	0

Stability and pH optimum

The activating metal of malate synthetase, Mg^{++}, has a strong influence upon the stability of the enzyme, particularly during ion exchange chromatography. In the absence of Mg^{++}, serious losses of activity occurred on DEAE-cellulose chromatography: this was largely prevented by the inclusion of $MgCl_2$ at 1 m*M* concentration during all stages of the purification, and by eluting the enzyme from DEAE-cellulose columns with $MgCl_2$.

The purified enzymes can be stored for at least a month at 2° in the presence of Mg^{++}, without loss of activity. The *Pseudomonas* enzyme is completely inactivated by freezing and thawing, but the yeast enzyme loses little or no activity by such treatment. It has been found repeatedly with both enzymes that, at specific activities higher than 5, serious inactivation occurs if fractionation with ammonium sulphate is attempted, even when Mg^{++} is present. It is possible that a high concentration of the ammonium cation displaces Mg^{++} and that the enzyme protein consequently becomes more susceptible to inactivation.

The pH-dependence of the activities of the two enzymes is seen in Fig. 4. Both enzymes are optimally active at pH 8.5, but the yeast enzyme has a broader range of activity on the acid side. Whilst the *Pseudomonas* enzyme is virtually inactive at pH 7.0, the yeast enzyme is still almost half as active as at its optimum.

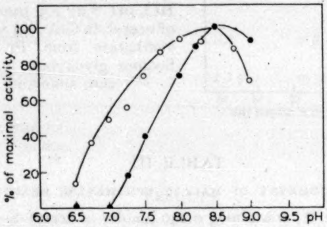

Fig. 4. Effect of pH on the activity of malate synthetase from baker's yeast (○—○) and *Pseudomonas ovalis* Chester (●—●). Each assay system contained 0.02 Units of malate synthetase; for other conditions see Fig. 3. All buffers were 0.1 *M* Tris·HCl.

Specificity of malate synthetase

The purified enzymes from both baker's yeast and *Pseudomonas* are specific for glyoxylate: no activity was detected with oxaloacetate, pyruvate, α-ketoglutarate, glyoxal, glycolaldehyde, formaldehyde or acetaldehyde. In the presence of glyoxylate there was no reaction with S,N-diacetyl β-mercaptoethylamine, S-acetyl pantetheine, propionyl coenzyme A or butyryl coenzyme A, but fluoroacetyl coenzyme A reacted at approximately one quarter of the rate observed with similar concentrations of acetyl coenzyme A. Malyl coenzyme A was not split by the enzyme either alone or in the presence of glyoxylate. It was not possible, however, to determine which carboxyl of malate was esterified in the CoA thiolester. The possible malyl CoA intermediate in the malate synthetase reaction would possess its hydroxyl group β- to the thiolester linkage and it is reasonable to assume that a proportion of the synthetic malyl CoA would be of this configuration. Since no hydrolysis of the synthetic malyl CoA was detected, it seems likely that malyl CoA is not an intermediate in the malate synthetase reaction.

Activators of malate synthetase

Purified malate synthetase requires Mg^{++} for activity. Mg^{++} could be only partially replaced by Mn^{++}, since at concentrations of Mn^{++} corresponding to the Mg^{++} concentrations required to saturate the enzyme, the enzyme began to be precipitated. Zn^{++}, Cu^{++}, Hg^{++}, and Co^{++} were ineffective and only in the case of Co^{++} could activity be restored by the addition of Mg^{++}.

Effect of Mg^{++} concentration

In Fig. 5 tracings of the progress curves of the malate synthetase reaction at increasing Mg^{++} concentrations are reproduced. It should be noted that the enzyme preparations used in this study were obtained from baker's yeast by the purification procedure described, but in which Mg^{++} was omitted. The activity of such preparations was lower and the enzyme was less stable than when Mg^{++} was present (see stability section). There is a pronounced lag period after the addition of Mg^{++}, which may be interpreted as the time required for Mg^{++} to be bound by the enzyme protein. If a reciprocal plot is made of velocity against (total) Mg^{++} concentration, then the apparent K_m is $5 \cdot 10^{-4}$ *M*. This value is obtained both in the presence of phosphate and Tris buffers.

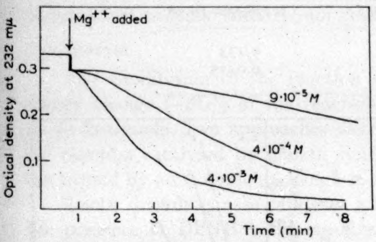

Fig. 5. Effect of Mg^{++} concentration on the velocity of the malate synthetase reaction. The reaction mixture contained, in 0.42 ml, 0.03 μmoles of acetyl-S-CoA; 40 μmoles of potassium phosphate, pH 7.6; 0.2 μmoles of sodium glyoxylate and 0.013 Units of Mg-free yeast malate synthetase. $MgCl_2$ to final concentrations as indicated was added at the point indicated by the arrow. (This figure is a tracing taken from a CARY recording spectrophotometer.)

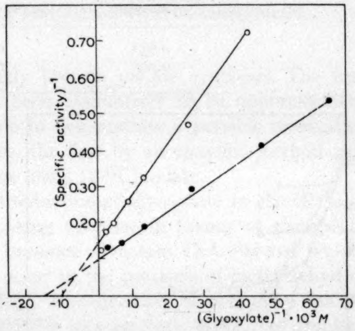

Fig. 6. LINEWEAVER-BURK plot of the effect of glyoxylate concentration on the initial rates of the reactions catalysed by the malate synthetase of baker's yeast (○—○) and of *Ps. ovalis* Chester (●--●).

Kinetic properties

The effect of glyoxylate concentration: When the glyoxylate concentration is varied in the presence of a constant concentration of acetyl CoA and Mg^{++}, simple MICHAELIS-MENTEN kinetics are followed: reciprocal plots[32] for both the baker's yeast and *Pseudomonas* enzymes are shown in Fig. 6. The K_m for glyoxylate at pH 8.0, 25° is for the yeast enzyme $9.3 \cdot 10^{-5}$ *M*, for the *Pseudomonas* enzyme $6.3 \cdot 10^{-5}$ *M*.

The effect of acetyl CoA concentration: The affinity of the purified enzyme for acetyl CoA is extremely high. There was no diminution in initial rate at the lowest concentration of acetyl CoA ($1.3 \cdot 10^{-5}$ *M*), for which the reaction was measurable (Fig. 7). The K_m for acetyl CoA ~~must~~ therefore be $\leqslant 1 \cdot 10^{-5}$ *M*.

It will be noted from Fig. 7 that there is a marked inhibitory effect at the highest acetyl CoA concentration used. This effect is much more pronounced in phosphate buffer than in Tris, and at acetyl CoA concentrations higher than 0.1 μmoles/ml the reaction is extinguished in phosphate buffer. This effect is illustrated in Table IV, where a comparison is made of the initial reaction rates in the two buffers. Experiments with unlabelled acetyl-S-CoA and with [1-^{14}C]acetyl-S-CoA showed that this effect was not due to an alteration in the course of the reactions: in either buffer malate was the only product formed (*cf.* Table III A).

The highest specific activity of malate synthetase achieved by the present purification procedures was 12.1 (see Table Ic). This represents 12.1 μmoles acetyl–S–CoA split/min/mg protein at 25°. Since the molecular weight is at present unknown, the turnover number may be represented as 1210 moles/min/100,000 g of enzyme at 25°.

TABLE IV

INHIBITION OF MALATE SYNTHETASE BY EXCESS ACETYL COENZYME A IN THE PRESENCE OF PHOSPHATE

For reaction conditions see Table III, Expt. B.

Concentration of acetyl coenzyme A (μmoles/ml)	*Initial rate of cleavage (ΔE_{232}/min) in*	
	0.1 M Tris pH 7.8	*0.1 M Phosphate pH 7.8*
0.055	0.268	0.233
0.107	0.225	0.0415

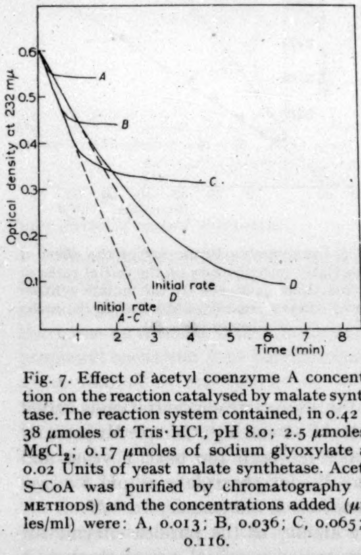

Fig. 7. Effect of acetyl coenzyme A concentration on the reaction catalysed by malate synthetase. The reaction system contained, in 0.42 ml, 38 μmoles of Tris·HCl, pH 8.0; 2.5 μmoles of $MgCl_2$; 0.17 μmoles of sodium glyoxylate and 0.02 Units of yeast malate synthetase. Acetyl–S–CoA was purified by chromatography (see METHODS) and the concentrations added (μmoles/ml) were: A, 0.013; B, 0.036; C, 0.065; D, 0.116.

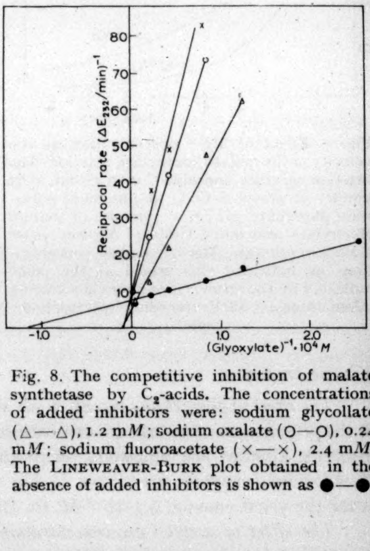

Fig. 8. The competitive inhibition of malate synthetase by C_2-acids. The concentrations of added inhibitors were: sodium glycollate (△—△), 1.2 m*M*; sodium oxalate (○—○), 0.24 m*M*; sodium fluoroacetate (×—×), 2.4 m*M*. The LINEWEAVER-BURK plot obtained in the absence of added inhibitors is shown as ●—●.

This figure may be compared to a turnover number of 4600 moles/min/100,000 g protein at 25° obtained by OCHOA[33] for crystalline condensing enzyme. The enzyme partially purified by WONG AND AJL[1] had a turnover number calculated in the above terms of 253 moles/min/100,000 g at 33°.

Inhibitors

The purified enzymes are strongly inhibited by several C_2-acids: the kinetics of the inhibitions (Fig. 8) show that this inhibition is strictly competitive for glyoxylate.

Oxalate is the most powerful inhibitor ($K_i = 1.9 \cdot 10^{-5}\ M$), followed by fluoroacetate ($K_i = 2.46 \cdot 10^{-4}\ M$) and glycollate ($K_i = 3.08 \cdot 10^{-4}\ M$). Malate synthetase is not inhibited by acetate, glycolaldehyde or oxaloacetate. L-malate was a weak non-competitive inhibitor. This suggests that the requirements for competitive inhibition include a carbon chain of 2 units and a carboxyl group. Acetate is ineffective but substitution of the methyl group by fluorine or –OH confers inhibitory power while substitution of methyl by carboxyl produces a powerful inhibitor having an affinity almost five times higher than that of the substrate, glyoxylate. The concentration at which oxalate produces its effect is 16 times lower than the Mg^{++} concentration in the reaction mixture, so that its inhibitory effect cannot be through complex formation with Mg^{++}.

Compounds reacting with –SH groups, such as iodoacetate and N-ethyl maleimide, do not affect enzymic activity, nor is activity stimulated by thiol compounds.

Equilibrium

The equilibrium of the reaction strongly favours malate synthesis. The free energy change ($-\Delta G^\circ$) of the reaction has been calculated[34] to be approximately 10–12 kcal/mole. Two approaches were made to demonstrate a possible reversal of the reaction catalysed by malate synthetase, the first by an enzymic method and the second by an isotopic exchange technique using [1-^{14}C]malate.

Lactic dehydrogenase catalyses a rapid reduction of glyoxylate to glycollate in the presence of DPNH[35], the equilibrium being very far in favour of glyoxylate reduction. If glyoxylate were formed in the presence of malate, CoA–SH and malate synthetase, an oxidation of DPNH should occur in the presence of lactic dehydrogenase. This was tested by incubating a high concentration of potassium malate (800 µmoles) with CoA–SH (1.3 µmoles), DPNH (0.4 µmoles), $MgCl_2$ (10 µmoles), potassium phosphate pH 7.6 (100 µmoles), crystalline lactic dehydrogenase and purified malate synthetase in a final volume of 3.1 ml. No decrease of optical density at 340 mµ was detected and hence no oxidation of DPNH was observed.

The second and more sensitive approach involved the use of [1-^{14}C]malate prepared by the action of malate synthetase upon acetyl–S–CoA and [1-^{14}C]glyoxylate (see METHODS). The reaction mixture (2.97 ml) contained acetyl–S–CoA (0.25 µmoles), an excess of sodium glyoxylate (5 µmoles), [1-^{14}C]malate (0.157 µmoles, giving $2.9 \cdot 10^5$ counts/min under the conditions of radioassay used), $MgCl_2$ (10 µmoles), Tris·HCl buffer, pH 8.0, (260 µmoles), and 0.6 units of yeast malate synthetase. A control tube contained no malate synthetase, but all other constituents. After 30 min, the reaction was stopped by the addition of 1.0 ml of 0.1 % 2,4-dinitrophenylhydrazine in 2 N HCl and the mixture was left for 10 min at room temperature. The aqueous solution was then extracted with benzene (glyoxylate 2,4-dinitrophenylhydrazone is quantitatively removed by this procedure) and portions of the benzene solutions were plated onto stainless steel planchets. There were 200 counts/min in the benzene layer from the complete reaction mixture and 230 counts/min from the control. Since there were $2.9 \cdot 10^5$ counts/min in the [1-^{14}C]malate added, no measurable reversal of the malate synthetase reaction had occurred under these conditions.

Exchange between CoA[^{35}S]H and Acetyl–S–CoA

A study of the exchange reaction,

$$\text{CoA}[^{35}\text{S}]\text{H} + \text{Acetyl–S–CoA} \rightleftharpoons \text{Acetyl}\ [^{35}\text{S}]\text{CoA} + \text{CoA–SH}$$

was deemed of interest in order to examine the possibility that an acetyl-group from acetyl CoA was bound to malate synthetase as an intermediate stage in the reaction of acetyl CoA with glyoxylate, thus:

$$\text{Acetyl}\cdot\text{S}\cdot\text{CoA} + \text{Enzyme H} \rightleftharpoons \text{Ac-Enzyme} + \text{CoA–SH} \tag{3}$$

$$\text{Ac-Enzyme} + \text{Glyoxylate} + H_2O \longrightarrow \text{Malate} + \text{Enzyme-H} \tag{4}$$

To this end CoA[^{35}S]H was prepared biosynthetically (as described under METHODS). It was found that CoA–SH and Acetyl–S–CoA were not easily separated, either in the solvent of STADTMAN[20] or by ionophoresis[29]; but, if the CoA–SH were alkylated with iodoacetate, then S-carboxymethyl CoA was easily separated from acetyl–S–CoA in a solvent consisting of ethanol–0.1 M potassium acetate (60:40), pH 4.5. Control experiments showed that acetyl–S–CoA was not attacked by iodoacetate under these conditions. After separation of S-carboxymethyl CoA and acetyl–S–CoA, the compounds were located as quenching spots under u.v. light, and were eluted onto glass cover slips and counted in a gas flow counter operating in the proportional range. Table V shows the extent of exchange under the two sets of experimental conditions.

TABLE V

EXCHANGE OF CoA^{35}SH WITH ACETYL–S–CoA

Expt.	*Radioactivity (counts/min) in*		*% Exchange*
	S-carboxymethyl CoA	*Acetyl-S-CoA*	
Complete A	381	75	33
No enzyme A	374	73	32.8
Complete B	403	169	59
No enzyme B	529	191	53

Expt. A. The reaction mixture contained, in a final volume of 0.16 ml 0.034 µmoles of CoA^{35}SH (reduced by borohydride), 0.020 µmoles of acetyl–S–CoA, 10 µmoles of Tris·HCl buffer pH 8.0, 1 µmole of $MgCl_2$ and 0.02 Units of malate synthetase (yeast). Expt. B. The reaction mixture was similar to that of Expt. A with the exception that borohydride-reduced CoA^{35}SH was replaced by a mixture of 0.25 µmoles of freshly prepared CoASH and 0.054 µmoles of CoA·^{35}S–^{35}S–CoA. The reaction was stopped after 15 min by the addition of 5 µmoles of iodoacetate. The mixtures were allowed to stand for 2 h at room temperature, acidified with 0.02 ml of glacial acetic acid, evaporated under N_2 at 40° and chromatographed on Whatman No. 3 paper.

It may be noted that a non-enzymic ester interchange occurs. This exchange reaction has already been described by WIELAND AND KÖPPE[36]. Within experimental error, there was no catalytic effect of malate synthetase upon the exchange, and hence no evidence for the formation of an acyl enzyme as an intermediate in the malate synthetase reaction was obtained.

DISCUSSION

Malate synthetase catalyses the condensation of acetyl–S–CoA and glyoxylate to form malate and CoA–SH, a water molecule being taken up in the reaction[1]. A carbon-carbon bond is formed between the methyl carbon of the acetyl group of acetyl–S–CoA and the carbonyl of glyoxylate, and a carbon–sulfur bond is broken. This enzyme, therefore, catalyses a reaction closely similar to that of the citrate-forming condensing enzyme[2]. Several differences, however, are apparent between the two enzymes.

First, malate synthetase (unlike condensing enzyme[33]) is dependent for its activity on the presence of Mg^{++}. In view of the closely analogous reactions catalysed by the two enzymes, and in the absence of published evidence to the contrary, it might seem fruitful to examine condensing enzyme for the possible presence of Mg^{++} tightly bound to the enzyme protein.

The second major difference lies in the distribution of the enzymes. Whilst condensing enzyme appears to be widely distributed among mammalian, microbial and plant systems, malate synthetase appears to be absent from animal tissues[3].

In contrast to condensing enzyme, it has not so far proved possible to demonstrate reversal of the reaction catalysed by malate synthetase: enzymic and isotopic exchange techniques similar to those effective with condensing enzyme gave negative results with malate synthetase. This less easy reversal is consistent with the larger decrease of free energy associated with malate formation: whilst the ΔG° for the synthesis of citrate from acetyl–S–CoA and oxaloacetate is —7.5 kcal/mole, BURTON[34] has calculated that ΔG° for the synthesis of malate from acetyl–S–CoA and glyoxylate is of the order of 10–12 kcal/mole.

The mechanism of the reaction catalysed by malate synthetase is probably very similar to that of the condensing enzyme, and is likely to involve an activation of the methyl carbon of acetyl–S–CoA by which a nucleophilic attack is made by this carbon on the carbonyl of glyoxylate, producing a new carbon–carbon bond in an aldol condensation.

Two aspects of the mechanism of the malate synthetase reaction have been studied. Since a molecule of water is involved in the condensation, it seemed possible that the reaction might proceed via the sequence:

$$\text{acetyl–S–CoA} + \text{glyoxylate}^- \longrightarrow \text{malyl–S–CoA}^- \tag{5}$$

$$\text{malyl–S–CoA}^- + H_2O \longrightarrow \text{malate}^{--} + H^+ + \text{CoASH} \tag{6}$$

However, synthetic malyl–S–CoA was not split by the enzyme, either in the presence or absence of glyoxylate. As noted above, it was not known that the compound tested was the correct isomer of malyl–S–CoA (*i.e.* $^-$OOC–CHOH–CH_2–CO–S–CoA), but it is reasonable to assume that the product of the reaction of malic anhydride with CoA–SH would contain enough of this material for its hydrolysis to be observed, if indeed it were an intermediate in the reaction. That no hydrolysis was detected therefore suggests that malyl–S–CoA is not an intermediate in the formation of malate.

The second aspect of the reaction mechanism studied concerned the possibility that the acetyl group of acetyl–S–CoA was reversibly bound to the enzyme in the absence of glyoxylate, thus:

$$\text{acetyl–S–CoA} + \text{enzyme}\cdot\text{H} \rightleftharpoons \text{acetyl}\cdot\text{enzyme} + \text{CoA–SH} \tag{3}$$

It was found that the rate of incorporation of radioactivity from $CoA^{35}SH$ into unlabelled acetyl–S–CoA was unaffected by the presence of enzyme, and it is therefore probable that the methyl group of acetyl–S–CoA is activated whilst still in combination with coenzyme A.

Recent studies[37,38] of the mechanism of reaction of condensing enzyme have shown that this enzyme cannot activate the methyl group of acetyl–S–CoA (as measured by exchange of D_2O[37] or TOH[38] with the methyl hydrogens) in the absence of the second reactant, oxaloacetate. If, as seems likely, a similar situation exists with malate synthetase, the labilization of a hydrogen from the methyl group may depend, not only on its proximity to the carbonyl of a thiol ester[39], but also on the pull exerted by the adjacent carbonyl of glyoxylate. It is conceivable that the electrophilic character of the carbonyl carbon of glyoxylate is enhanced by the presence in its vicinity of positively-charged groups on the enzyme surface. As the carbon-sulfur bond is broken, the new carbon–carbon bond would be simultaneously formed. The function of Mg^{++} in this reaction might be to form a linkage between either glyoxylate or acetyl–S–CoA and the enzyme. In view of the apparently obligatory requirement for Mg^{++} in enzymic reactions involving ATP, it seems more likely that Mg^{++} is involved in the binding of the adenosine polyphosphate portion of acetyl–S–CoA to the enzyme.

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SUBSTRATE SPECIFICITY OF *SACCHAROMYCES FRAGILIS* GALACTOKINASE

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SUMMARY

The substrate specificity of *Saccharomyces fragilis* galactokinase has been investigated using a series of compounds structurally related to galactose. Only those compounds, which, having the α-pyranose configuration, differ from galactose at the level of C2 (2-deoxygalactose, galactosamine, talose) have been found to be phosphorylated. Changes at C3, C4 or C6 (gulose, glucose, L-arabinose, D-fucose, D-glycero-D-galactoheptose) result in compounds towards which this enzyme is inactive. The effect of the concentration of ATP on the activity of the enzyme has also been explored.

INTRODUCTION

Galactokinase was discovered independently and almost simultaneously by **Leloir** and collaborators[1] and by **Willkinson**[2]. Some general properties of the enzyme from yeast and mammalian organs have been described (see ref. 3), but very little is known about its substrate specificity. **Willkinson** has shown that tagatose is not phosphorylated by the yeast enzyme[2]. CARDINI AND LELOIR described the phosphorylation of galactose and galactosamine by yeast, brain and liver extracts[4]; however, the identity of the enzyme(s) catalyzing these phosphorylations was not conclusively established.

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The characterization of the substrate specificity of the galactokinase of adapted *Saccharomyces fragilis* was of interest to us, not only from a general point of view, but also because of its relationship to studies undertaken in our laboratory on the mechanism of transport of galactose into the intact yeast cell[5].

In this work an experimental approach was used similar to that of SOLS AND CRANE in their fundamental study of brain hexokinase[6]. For a more detailed theoretical discussion reference may be made to their paper. In order to establish which parts of the sugar molecule are essential for the enzyme–substrate complex formation, a series of compounds structurally related to galactose have been assayed. It appears that galactokinase has a rather narrow spectrum of specificity. Both *apparent* affinity and maximal velocity decrease when modifications are introduced at the level of the second carbon atom of the galactose molecule (2-deoxygalactose, galactosamine, talose), but the resulting compound is still phosphorylated. Modifications of galactose at carbon atoms 3, 4 or 6 (gulose, glucose, L-arabinose, fucose, D-glycero-D-galactoheptose) were associated with both a disappearance of measurable phosphorylative capacity by galactokinase and the absence of detectable inhibitory action on galactokinase. The effect of adenosinetriphosphate (ATP) concentration on the reaction rate has also been investigated.

MATERIALS AND METHODS

This work was carried out with a strain of *Saccharomyces fragilis* obtained from the Instituto Jaime Ferrán de Microbiologia at Madrid. The yeast was grown in Roux bottles containing a yeast extract–galactose medium* (2 % galactose).

Galactokinase was extracted** and purified as described by TRUCCO *et al.*[1]. Further purification and elimination of contaminating hexokinase are described in the text.

Compounds used. Talose and D-glycero-D-galactoheptose were obtained from Dr. N. K. RICHTMYER; tagatose and 2-deoxygalactose from Light. Calcium gulonate was from General Biochemicals, Inc.; calcium was removed by means of potassium phosphate, pH 7. Galactose, galactosamine, dulcitol, L-arabinose, fructose and glucose were obtained from Pfanstiehl. Galactose was treated with baker's yeast in order to remove contaminating glucose. L-arabinose was recrystallized from alcohol until its contaminating galactose content decreased below 0.2 %. Galactosamine hydrochloride was neutralized with KOH before use. All sugars were allowed to attain mutarotation equilibrium. Excepting L-arabinose, all the sugars belonged to the D-series.

ATP was obtained from Sigma. Ethylenediaminotetraacetate (Versene) was the disodium salt supplied by the Bersworth Chemical Co. Bentonite from Tidinit was washed with HCl and neutralized before use.

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* I wish to express my gratitude to Dr. R. BELTRÁ for her help in the preparation of the cultures.

** Crude preparations contained 4,000 galactokinase and 10,000 hexokinase units (as measured on glucose) per gram of dried yeast.

Enzymic assays. Two methods were principally used; the colorimetric indicator method of Sols and Crane[6] and the reducing power disappearance method[1]. In this latter case, barium-zinc filtrates[7] of the incubation mixtures were analyzed for reducing power with the Somogyi method[8] for common sugars, or with the Shaffer–Somogyi method for deoxysugars[9]. In the case of galactokinase, since the reaction product is a non-reducing ester, Ba–Zn treatment can be omitted, as shown in Fig. 1. The reaction may be stopped with Versene[10], residual reducing power being directly estimated in aliquots. The ATP and protein at the concentrations used do not interfere. If a crude preparation of enzyme is used, protein must be eliminated.

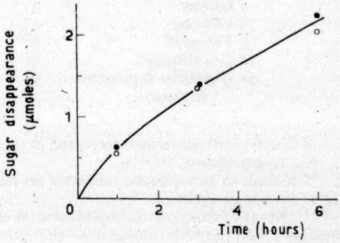

Fig. 1. Phosphorylation of talose. 5 μmoles of talose, 7.5 μmoles of ATP, 3.75 μmoles $MgSO_4$, 10 μmoles fluoride, 25 μmoles pH 7 maleate buffer, and 25 units of enzyme were incubated at 30° in a final volume of 0.4 ml. Series ○ was treated with Ba–Zn[1]. Series ● was treated with Versene[10]. Residual or free talose was estimated in aliquots[8].

An enzyme unit is defined as the amount of enzyme which can catalyze the phosphorylation of 1 μmole of hexose in 15 min of incubation at 30°.

Protein was estimated according to Lowry *et al.*[11]. If not stated otherwise, assays were carried out at pH 6.4–7.2[1] and in the presence of saturating concentrations of ATP and Mg (ratio ATP/Mg = 2). Fluoride was used in the incubation mixtures; no trace of interfering phosphatases was observed.

EXPERIMENTAL

In Table I, the Michaelis constants, relative maximal rates and phosphorylation coefficients of the compounds tested are specified.

Evaluation of Michaelis constants. When possible (compounds 1, 4, 5 and 6, Table I), Michaelis constants were measured directly by studying the effect of substrate concentration on reaction rate; Lineweaver and Burk plots[12] were used for the calculations. The colorimetric indicator method was employed whenever possible (all compounds except 4 in Table I) because of its sensitivity. The effect of varying concentrations of substrate on phosphorylation rate was studied at a constant volume. Galactosamine required a special approach, which is described later.

Minimal values for the inhibition constants of different compounds were calculated from their inhibitory effect on galactose phosphorylation, using either the colorimetric indicator method (dulcitol, tagatose) or the reducing power disappearance method (dulcitol).

Relative maximal velocities. $V_{\max}$ for galactose was measured directly at saturating concentrations of substrate. For other substrates (compounds 4, 5 and 6, Table I), maximal velocities were calculated from the experimental data. A *minimal value* for the concentrations used was calculated in the case of the apparently inert compounds (those labeled with a dash, column 4, Table I).

TABLE I

SUBSTRATE SPECIFICITY OF *Saccharomyces fragilis* GALACTOKINASE

Compound	*Modified at carbon*	K_m* *moles/l*	*Relative maximal rate***	*Phosphorylation coefficient****
1 Galactose		$3 \cdot 10^{-4}$	1	1
2 Dulcitol	1	—	—	—
3 Tagatose	1, 2	—	—	—
4 Galactosamine	2	$1 \cdot 10^{-2}$§	0.4	$1.2 \cdot 10^{-2}$
5 2-deoxygalactose	2	$2 \cdot 10^{-2}$	0.4	$6 \cdot 10^{-3}$
6 Talose	2	$2 \cdot 10^{-1}$	0.1	$1.5 \cdot 10^{-4}$
7 Gulose	3	—	—	—
8 Glucose	4	—	—	—
9 Fucose§§	6	—	—	—
10 L-arabinose	6	—	—	—
11 D-glycero-D-galacto-heptose	6	—	—	—

* The K_m values are not corrected for mutarrotation equilibria. A dash in this column indicates a K_m bigger than $5 \cdot 10^{-1}$ *M*.

** A dash in this column indicates an undetectable velocity (less than 0.01 at a concentration of $1 \cdot 10^{-2}$ *M* or greater).

*** A dash in this column indicates that the compound appeared to be inert both as a substrate and as a competitive inhibitor to the extent that it cannot have a phosphorylation coefficient greater than $5 \cdot 10^{-5}$.

§ At pH 8; see the text.

§§ Unpublished observations of Dr. C. F. DE HEREDIA.

Phosphorylation coefficients were calculated from the K_m and maximal rate values[6], or a minimal figure was calculated[10] in those cases where no appreciable phosphorylation or inhibitory effects could be observed.

Test for false positives. Since most available sugars contain variable amounts of other sugars as impurities, experiments were carried out to detect any positive results that could be erroneous. At an average concentration of $1 \cdot 10^{-2}$ *M*, every presumptive substrate was incubated with ATP–Mg and an excess of enzyme long enough to assure the close approach to reaction completion. The following percentages of phosphorylation were obtained: 2-deoxygalactose, over 95; galactosamine, 90; talose, 70. With the other compounds tested, the disappearance of reducing power was less than 5 %.

Glucose. To study glucose as a presumptive substrate, it was necessary to obtain galactokinase preparations essentially free of hexokinase. This was accomplished by adsorbing the interfering enzyme on pH 6.4 bentonite (100 mg/mg protein). The relative rates of phosphorylation of glucose, fructose and galactose in the crude extracts were 1:1.5:0.4, whereas in the more purified preparations they were 1:1.5:17. The ratio glucose:fructose is similar to that found in a variety of yeasts[13]. The fact that this ratio did not change after the relative concentration of galactokinase (about 40 times) suggests that the phosphorylation of both glucose and fructose is due only to one enzyme, namely, hexokinase. The negligible affinity of galactokinase for glucose is also evidenced by the lack of appreciable inhibitory effect of glucose on galactose phosphorylation, even at the enormous glucose concentration used (more than 400 times the concentration of galactose; see Fig. 3).

Galactosamine. The modified reducing power disappearance method has provided an especially useful tool for the study of galactosamine phosphorylation. Neither the normal colorimetric indicator method nor the reducing power disappearance method can be employed in this special case, since galactosamine is variably ionized at the pH range 6 to 8 and since free aminosugars are partially adsorbed on the barium sulfate gel. A constant amount of galactosamine was incubated (conditions explained in Fig. 2) with the enzyme and an excess of ATP–Mg, the substrate concentration being varied by increasing with water the total volume of the incubation mixtures. That velocity changes are not due to the dilution of ATP, Mg or enzyme, is supported by the lack of unspecific dilution effects on the rate of phosphorylation of a substrate with high affinity, as galactose. The K_m of galactosamine rises with pH from a minimum below pH 6.5 to a maximum above pH 8. Preliminary results indicate that the increase in apparent affinity describes an S-shaped curve which can be superimposed on the galactosamine hydrochloride titration curve. As it happens in the case of hexokinase and glucosamine[6] only the non-ionized form of the aminosugar seems able to combine with the enzyme, the galactosammonium ion being inert.

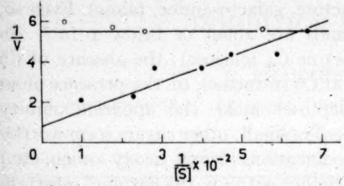

Fig. 2. Effect of galactosamine concentration on velocity. ●: 5 μmoles galactosamine, 7.5 μmoles ATP, 3.75 μmoles $MgSO_4$, 200 μmoles tris(hydroxymethylaminomethane) pH 8 and 6.7 galactokinase units were incubated for an hour at 30°. The final volume of the incubation mixtures ranged from 0.25 to 2.65 ml. Reactions were stopped with Versene[10] and volumes completed to 5 ml. Residual galactosamine was determined in 1 ml aliquots[8]. Velocity and concentration are plotted as reciprocals[12]. A parallel experiment was performed with galactose (○) instead of galactosamine (6.7 galactokinase units, six minutes of incubation at pH 7).

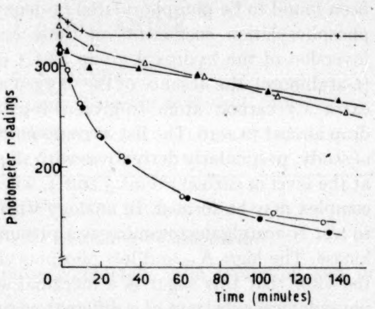

Fig. 3. Glucose as a presumptive substrate. Photometric indicator method. Galactokinase preparation, described in the text. ▲, 1 *M* glucose; △, 0.005 *M* fructose; ○, 0.0025 *M* galactose; ●, 0.0025 *M* galactose plus 1 *M* glucose; + blank without sugar.

Influence of ATP concentration. This has been studied by using a colorimetric mixture containing $3.7 \cdot 10^{-3}$ *M* Mg^{++} and galactose at a concentration more than ten times above K_m. Reactions were started by adding variable amounts of ATP. At a concentration of ATP of $1 \cdot 10^{-4}$ *M*, velocity was about half maximal or less.

DISCUSSION

It seems clear that, since the galactokinase reaction product is the Kosterlitz ester, α-D-galactopyranose-1-phosphate[1,2,14] phosphorylation takes place through the glycosidic hydroxyl of the α-pyranose ring. This is supported by the fact that compounds closely related to galactose, but lacking the adequate configuration, have undetectable affinity (dulcitol, tagatose). A compound such as the 1,5-anhydrogalactitol, which has the same configuration as galactopyranose, but lacks the hydroxyl group necessary for phosphorylation, might be expected to be a strong competitive inhibitor.

Galactokinase seems to have a rather narrow spectrum of specificity, since only compounds which differ from galactose by their configuration at carbon atom 2 have been found to be phosphorylated (2-deoxygalactose, galactosamine, talose). Even so, phosphorylation coefficients of these compounds are about or below $1 \cdot 10^{-2}$. An inversion of the hydroxyl group at C3 (gulose) or C4 (glucose); the absence of C6 (L-arabinose), the absence of the oxygen atom at C6 (D-fucose), or the presence of an extra C7 carbon atom (D-glycero-D-galactoheptose) make the apparent affinity drop almost to zero. The list of compounds tested is small; other sugars seem worthy of study, particularly derivatives with slight modifications (deoxy, deoxy-amino, etc.) at the level of carbon atoms 3 and 4, where, together with C6, the enzyme–substrate complex may be formed. In analogy with hexokinase, it will probably be interesting to test N-acetylgalactosamine as a presumptive competitive inhibitor of the galactokinase. The high K_m and low phosphorylation coefficient of galactosamine supports the view that this sugar is a marginal substrate of galactokinase, rather than the physiological substrate of a different enzyme.

The *apparent* K_m of ATP is similar in the case of both galactokinase and hexosekinase; similar value has been found for the hexokinase of *S. cerevisiae*[10].

ACKNOWLEDGEMENTS

I wish to thank Dr. A. SOLS for his interest and advice in this investigation, and Dr. K. R. HANSON for his valuable criticism of the manuscript.

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RESTAURATION DE LA SYNTHÈSE D'ENZYMES APRÈS INHIBITION PAR L'AZAGUANINE

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SUMMARY

Restoration of enzyme synthesis after inhibition by azaguanine

Azaguanine inhibits protein synthesis almost completely. The inhibition can be released by guanosine.

The later guanosine is added, the more imperfect becomes the restoration of protein synthesis.

The damages caused by azaguanine affect more severely the formation of certain enzymes than that of others.

It is concluded that azaguanine interferes with the activity of substances which are involved in the control of specificity in protein synthesis.

INTRODUCTION

La 8-azaguanine inhibe fortement toute synthèse de protéines chez *Bacillus cereus*[1-4]. La guanosine peut lever l'inhibition si elle est ajoutée assez tôt après l'analogue. Nous avons noté brièvement ailleurs[5,6] que la synthèse de la pénicillinase se rétablit beaucoup plus tardivement que celle de l'ensemble des substances protéiques.

On trouvera ci-dessous des données nouvelles sur le comportement de deux enzymes, la pénicillinase et la catalase, lorsque la synthèse des protéines se rétablit sous l'action de la guanosine chez des bactéries intoxiquées par la 8-azaguanine.

MATÉRIEL ET MÉTHODES

Bactérie

Bacillus cereus: Souche produisant la pénicillinase comme enzyme constitutif. Ce mutant fut isolé d'une souche de *B. cereus* NCTC 569 selon KOGUT *et al.*[7].

Dosage de la pénicillinase

Adaptation de la méthode de PERRET[8]. La synthèse de l'enzyme était arrêtée par addition de 0.1 ml d'une solution alcoolique 0.01 M de 8-hydroxyquinoléine à 1 ml de suspension bactérienne. Un volume convenable (50 ou 100 μl) de la suspension était mélangé à 5 ml d'une solution de pénicilline dans un tampon de phosphate à pH 6.5 maintenu en thermostat à 30°. Des échantillons étaient prélevés au cours du temps et mélangés à 2 ml de la solution d'iode. L'excès d'iode était titré 10 min plus tard à l'aide de thiosulfate. Sous cette forme, la méthode est très précise; la réaction obéit à une cinétique d'ordre zéro. Unité d'activité calculée selon PERRET[8].

Dosage de la catalase

Des prises de 2 ml de suspension bactérienne étaient refroidies dans un bain de glace, centrifugées à froid, et le milieu était rejeté. Les bactéries étaient ensuite lavées plusieurs fois puis remises en suspension dans 2 ml de tampon de phosphate 0.02 M pH 7.0 et exposées pendant 60 sec aux ultrasons dans un bain de glace (appareil MSE-Mullard, 20 kc). Des essais systématiques avaient montré que la catalase est complètement accessible à son substrat après un tel traitement. L'activité des suspensions était ensuite déterminée selon VON EULER ET JOSEPHSON[9]. Les chiffres indiqués représentent les valeurs de la constante de vitesse de la réaction

$$k = 1/t_{min} \log_{10} a_0/a_t$$

obtenues quand 1 ml de suspension était mélangé à 20 ml d'une solution $7 \cdot 10^{-4}\ M$ de H_2O_2 dans un tampon de phosphate $10^{-2}\ M$, pH 7.0 maintenu dans un bain de glace fondante.

Autres techniques et conditions expérimentales: Voir publications antérieures[3, 4].

RÉSULTATS EXPÉRIMENTAUX

Rétablissement de la synthèse des protéines et des enzymes

Si de l'azaguanine est ajoutée à raison de 36 μg/ml à une suspension de *B. cereus* (0.3 mg/ml) en croissance exponentielle, il faut environ 10 min pour que l'inhibition

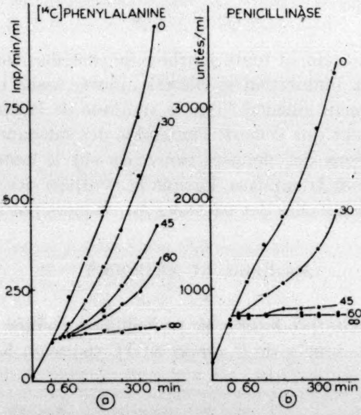

Fig. 1. Restauration de la synthèse de pénicillinase et de celle des protéines. Une suspension de *B. cereus* en croissance exponentielle dans un hydrolysat de caséine (20 mg/ml) contenant 0.08 μC de L-[^{14}C]phénylalanine/ml est divisée en cinq parties égales et agitée en thermostat à 30°. De l'azaguanine est ajoutée (36 μg/ml) à chaque fiole au temps 0. On ajoute de la guanosine (135 μg/ml) aux différentes suspensions bactériennes, respectivement 0, 30, 45 et 60 min plus tard. La dernière ne reçoit pas de guanosine.

a. ^{14}C incorporé dans les protéines (coups/min/ml suspension).

b, Pénicillinase constitutive (unités/ml).

de la synthèse des protéines s'exprime pleinement, dans les conditions de nos expériences[2-4]. Tout effet de l'inhibiteur peut être empêché par l'addition simultanée de guanosine (135 μg/ml).

La Fig. 1a montre comment se rétablit la synthèse des substances protéiques lorsqu'on ajoute la guanosine 30, 45 ou 60 min après l'azaguanine.

Il est clair que la formation de protéines reprend rapidement une allure à peu près normale si on ajoute la guanosine jusqu'à 30 min après l'inhibiteur. Mais si la guanosine est ajoutée plus tard, la synthèse de substances protéiques se rétablit d'autant plus lentement que le nucléoside normal est ajouté plus tard.

Dans cette expérience, la synthèse de substances protéiques a été mesurée par la quantité de L-phénylalanine incorporée dans le précipité trichloracétique[4]. Des résultats tout à fait semblables ont été obtenus en dosant les protéines selon LOWRY *et al.*[10].

La Fig. 1b indique comment la synthèse de pénicillinase constitutive s'est rétablie dans la même expérience. On remarquera que lorsque la guanosine était ajoutée 45 ou 60 min après l'azaguanine, la synthèse de la pénicillinase n'avait guère repris à un moment où la synthèse de substances protéiques s'était bien rétablie et se poursuivait déjà presqu'à la vitesse normale. Le dommage causé par l'azaguanine à la synthèse de la pénicillinase est donc plus sévère et plus difficile à réparer que le dommage causé à la synthèse des substances protéiques en général, et il s'accentue rapidement quand l'action de l'azaguanine se prolonge.

Tous les enzymes ne se comportent cependant pas comme la pénicillinase. Dans l'expérience dont les résultats sont rassemblés dans la Fig. 2, nous avons étudié

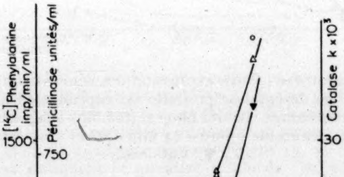

Fig. 2. Restauration de la synthèse de pénicillinase, de catalase et des substances protéiques. Une suspension de *B. cereus* en croissance exponentielle dans un milieu contenant de l'hydrolysat de caséine (20 mg/ml) et 0.08 μC de L-[^{14}C]phénylalanine/ml, est partagée en deux volumes égaux et agitée en thermostat à 30°. A l'une des suspensions bactériennes, qui sert de témoin (points blancs) on ajoute 36 μg d'azaguanine et 135 μg de guanosine simultanément au temps 0. A l'autre (points noirs) on ajoute l'azaguanine au temps 0 et la guanosine 45 min plus tard. ○, ●, ^{14}C incorporé dans les protéines; △, ▲, Pénicillinase formée; ▽, ▼, Catalase formée.

simultanément le rétablissement de la synthèse de la catalase, de la pénicillinase, et des protéines en général (incorporation de L-[^{14}C]phénylalanine) dans une même suspension de *B. cereus*. La guanosine fut ajoutée 45 min après l'azaguanine. Les échelles d'ordonnées ont été ajustées de façon à faire coïncider au mieux les courbes correspondant au témoin.

Comme dans l'expérience précédente, la synthèse de pénicillinase s'est rétablie plus tardivement et plus péniblement que celle des substances protéiques en général. La synthèse de la catalase, au contraire, a repris presqu'en même temps que l'incorporation de la phénylalanine. La Fig. 3 donne d'ailleurs une autre représentation des mêmes résultats: l'accroissement des enzymes y est exprimé en fonction de l'augmentation des substances protéiques (^{14}C de L-phénylalanine incorporé). On voit bien que la synthèse de pénicillinase reste bloquée pendant que des quantités très considérables de protéines se forment. Au contraire, la production de catalase reprend très peu de temps après l'incorporation de phénylalanine, et le taux différentiel de synthèse de cet enzyme est alors nettement plus élevé que dans le témoin.

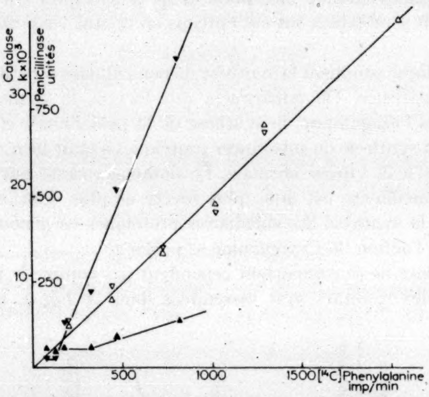

Fig. 3. Taux différentiel de synthèse. Autre expression des résultats de l'expérience de la Fig. 2. La quantité de pénicillinase ou de catalase produite est exprimée en fonction de la quantité de ^{14}C incorporé pendant le même temps. Points blancs, (témoin) azaguanine et guanosine ajoutées simultanément; points noirs, guanosine ajoutée 45 min après l'azaguanine. △, ▲: Pénicillinase. ▽, ▼: Catalase.

Expulsion de l'azaguanine des acides ribonucléiques

Les travaux de LATSNITZKI, MATTHEWS ET SMITH[11–13], MANDEL[14] et MARKHAM[15] ont établi que les purines normales ou leurs dérivés provoquent l'expulsion de l'azaguanine préalablement incorporée dans l'ARN.

La Fig. 4 montre qu'en effet une grande partie de l'azaguanine est expulsée assez rapidement lorsqu'on ajoute de la guanosine. Mais il semble bien qu'une fraction très notable de l'azaguanine soit fixée irréversiblement, ou qu'elle ne soit déplacée que très lentement par la guanosine.

On notera que la quantité d'azaguanine fixée, tant irréversiblement que d'une façon moins définitive, est d'autant plus grande qu'il s'écoule plus de temps entre l'addition de l'azaguanine et celle de la guanosine.

DISCUSSION

L'inhibition générale de la synthèse des protéines par l'azaguanine s'exprime complètement en 10 min[1,4]. L'azaguanine se substitue donc probablement à la guanine dans un métabolite qui se renouvelle rapidement[1,14] et dont la synthèse des protéines dépend. Le rétablissement rapide et complet des synthèses protéiques par la guanosine lorsque celle-ci est ajoutée peu de temps après l'analogue confirme cette manière de voir. La première substance importante affectée par l'azaguanine pourrait être par exemple l'acide guanosinetriphosphorique[14] ou bien un ARN à grande activité métabolique[1,4].

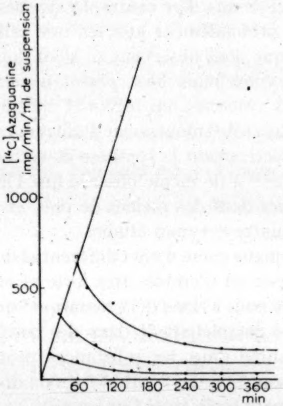

Fig. 4. Expulsion de l'azaguanine des acides nucléiques en présence de guanosine. Une suspension de *B. cereus* en croissance exponentielle est partagée en quatre parties égales dans des fioles qui sont agitées en thermostat à 30°. Au temps 0, de la 8-[2-^{14}C]azaguanine (4 μC/mg) est ajoutée à raison de 36 μg/ml à chaque fiole. La première fiole ne recevra aucune autre addition (points blancs). Aux autres, on ajoute 135 μg de guanosine respectivement 15, 30 et 60 min après l'azaguanine. Les points expérimentaux indiquent la quantité totale de [^{14}C]azaguanine contenue dans les acides nucléiques d'un ml de suspension.

Nos résultats indiquent qu'à côté de cette première action inhibitrice qui atteint la synthèse de toutes les protéines, des lésions plus profondes et plus lentes à guérir apparaissent lorsque l'action de l'azaguanine se prolonge; ces lésions secondaires n'affectent pas uniformément la synthèse de toutes les protéines. Il faut en conclure que l'incorporation prolongée, ou abondante, d'azaguanine détériore certaines substances dont la spécificité des synthèses dépend.

Il est raisonnable de penser que ces substances sont des acides ribonucléiques. L'accumulation progressive d'une fraction d'ARN dont l'azaguanine ne peut plus être expulsée par la guanosine (Fig. 4) pourrait être en relation avec l'irréversibilité croissante des dommages causés par l'azaguanine. Il faut noter toutefois que de très faibles quantités d'azaguanine s'incorporent dans les acides désoxyribonucléiques, et qu'on ne peut donc pas exclure à présent que les lésions secondaires se trouvent au niveau de ceux-ci.

Il y a plusieurs années, CREASER[16, 17] avait observé que l'azaguanine inhibe la synthèse induite de β-galactosidase chez un Staphylocoque sans inhiber la formation d'un système d'enzymes constitutifs, la glucozymase, ni celle de catalase constitutive. Ces résultats avaient fait croire que la synthèse induite d'enzymes serait sensible à l'azaguanine tandis que la formation des enzymes constitutifs ne le serait pas. Cette interprétation doit être abandonnée. En effet, la pénicillinase induite dans la souche de *B. cereus* NCTC 569 se comporte exactement comme la pénicillinase constitutive du mutant qui en dérive et avec lequel ont été effectuées toutes les expériences dont les résultats sont rapportés ci-dessus. Par contre, la synthèse de la pénicillinase constitutive est beaucoup plus profondément affectée que celle d'un autre enzyme, la catalase. L'effet différentiel que nous observons ne tient donc pas au caractère adaptatif ou constitutif des enzymes mais bien plutôt à certaines particularités des protéines elles-mêmes ou des systèmes qui régissent spécifiquement leur formation.

Les observations de CREASER[16, 17] montraient d'ailleurs très bien que chez *S. aureus* l'azaguanine peut inhiber sélectivement la synthèse de certains enzymes sans affecter la synthèse des autres. HEYES[18] a de même observé que l'analogue inhibe partiellement la synthèse des protéines dans des racines de pois, et que le degré d'inhibition n'est pas le même pour les quatre enzymes étudiés.

Chez *B. cereus*, on ne distingue guère d'effet différentiel de l'azaguanine au moment où l'inhibition s'établit: celle-ci est d'emblée très forte et elle atteint la synthèse de toutes les protéines. Toutefois nous avions déjà remarqué[4] que la synthèse de la pénicillinase peut être supprimée complètement dans des conditions où l'inhibition de l'incorporation de phénylalanine dans les substances protéiques n'est pas totale. Mais c'est au cours de la restauration des synthèses qu'on distingue le plus nettement des différences entre les enzymes (voir aussi CREASER[17]).

La raison pour laquelle certains enzymes sont plus profondément atteints que d'autres n'est pas évidente. Il est possible que les centres organisateurs de certaines protéines soient endommagés plus rapidement ou plus sévèrement que d'autres. On peut penser aussi que toutes les protéines formées pendant la phase de restauration sont anormales, mais que l'anomalie commune peut être ou non fatale à l'activité enzymatique, selon qu'elle affecte une partie essentielle de l'enzyme ou une région moins importante. Deux analogues de l'uracile, la 2-thiouracile[19] et la 5-fluorouracile[20] produisent des effets de cette sorte chez *E. coli*. C'est peut-être en déréglant le mécanisme de sélection des acides aminés que ces analogues, ainsi que l'azaguanine, perturbent la synthèse des enzymes[20, 21].

On remarquera enfin (Fig. 3) que le taux différentiel de synthèse de la catalase est plus élevé pendant la phase de restauration que pendant la croissance normale. A moins que la formation de catalase ne soit stimulée spécifiquement ou que l'enzyme formé ne soit plus actif que la catalase normale, cela indique que la synthèse de l'ensemble des substances protéiques — qu'elles soient parfaites ou non — n'est pas encore rétablie au moment où la formation de catalase est pleinement restaurée.

La synthèse des protéines ne se rétablit donc pas en bloc sous l'action de la guanosine. La restauration est progressive et les synthèses restent déréglées pendant plusieurs heures. L'assortiment des protéines formées pendant cette période est anormal; les protéines le sont peut-être aussi.

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SPECTROPHOTOMETRICAL INVESTIGATIONS ON ENZYME SYSTEMS IN LIVING OBJECTS

V. SPECTRAL TRANSMISSION OF YEAST SUSPENSIONS

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SUMMARY

Quantitative determination of enzymes in living material postulates a reliable reproduction of the shape of spectral bands and of a linear relation between extinction and number of molecules per unit volume. These conditions are approximately fulfilled at a medium density of a yeast suspension. Considerable deviations from linearity are observed at high densities, and to some extent also in very diluted suspensions. It is claimed that these deviations are in the former case caused by a partial total shading of the ingoing light and in the latter case by a rising percentage of light which does not pass through the interior of the cells. The bearing of these phenomen a on the apparent "flattening" of spectra of living material is discussed. The pronounced effect of salts is referred to their influence on the respiration and hence on the quantity of light absorbing material.

INTRODUCTION

The influence of the turbidity of living material on the spectrophotometric measurements has been much discussed[1-3, 16]. An appropriate treatment of the problem is of importance for calculation of the true concentration of respiratory enzymes and other pigments in living material. Turbid materials are changing a direct light beam into diffuse light. The relative intensity of that fraction of the in-going light which is measured by a photo tube placed at the opposite side of a turbid sample decreases with decreasing wave-length, but an exact calculation of these relations encounters difficulties. A simple procedure of eliminating some of the effects of light scattering is recording of difference spectra or else turning over the reference beam into diffuse light by means of a layer of filter paper or cotton wool introduced in the light path. This procedure was used in my work on the respiratory enzymes of wheat roots[4, 5], a fact overlooked by SHIBATA, BENSON AND CALVIN who later on presented a similar procedure under the heading "a new method"[6].

Because scattering of the reference beam by filter paper etc. can never quite imitate the effect of a turbid sample corrections must however be applied in order to obtain reliable calculations of the concentration of enzymes in living material. Some information about corrections to be made may be gained by observation of the band heights of known pigments, added to the samples, as compared with those in pure solutions[2]. Measurements in the region green-red as a rule need only little correction, whereas the correction factors are rapidly increasing in the blue-ultraviolet region. Because the degree of scattering varies with each investigated material correction factors have no general bearing but only individually calculated. The problem is still more complicated by the fact that the degree of light scattering and spectral transparency is influenced by the concentration of the suspensions, viz. the number of cells per unit volume, and by all treatments which affect the number, dimensions and shape of submicroscopical particles in the protoplasm. Due attention has also to be paid to comparatively rapid variations in the quantity of enzymes following upon addition of salts etc. These methodological problems were actualized during my recent investigations on the effect of salts on the steady states of the respiratory enzymes[7]. Suspensions of baker's yeast served as the experimental material.

Effects of varying concentration of suspensions

Washed baker's yeast was suspended in distilled water. The recipient (19 mm wide quartz tubes combined with a circulation pump, or quartz "circulation vessels" (see ref. 2, Fig. 2) of similar dimensions) was illuminated in vertical position by a 10 mm long and 4 mm wide horizontal light beam from a monochromator. The 10 × 4 mm wide opening of the photo multiplier (EMI quartz tube 6256 at approx. 1500 V) was fixed in a distance of about 3–5 mm from the recipient. As recorder served a sensitive Speedomax G. No difference in the results was observed if parallellepipedic recipients instead of cylindric ones were used. The recipients were gassed with pure nitrogen in order to keep the enzymes reduced. Complete spectra of α-, β- and γ-bands of cytochromes a, a_3, b and c and reduced diphosphopyridine nucleotide (DPNH) were recorded automatically[8]. The apparent concentrations of the enzymes was calculated according to a previously mentioned procedure[2].

Fig. 1 shows the direct spectrum of three concentrations (2.5, 5 and 10 % moist

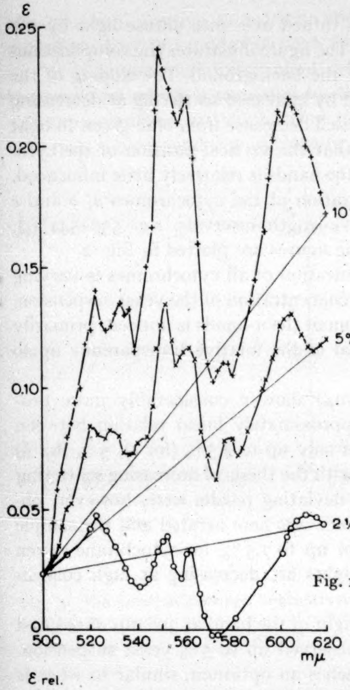

Fig. 1.

Fig. 1. Absorption spectrum of the α-β-region of the cytochromes in baker's yeast suspension of different concentration. Reference = 2–8 layers of colorless filter paper (Munktell No. 3).

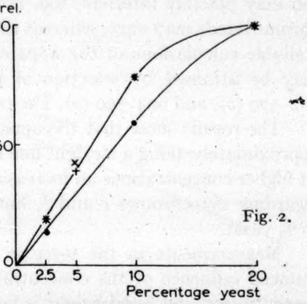

Fig. 2.

Fig. 2. Apparent concentration of the cytochromes *c*,*b* and *a*, calculated from the α-bands in Fig. 1. ●, cytochrome *a*; ×, cytogrome *c*; +, cytogrome *b*.

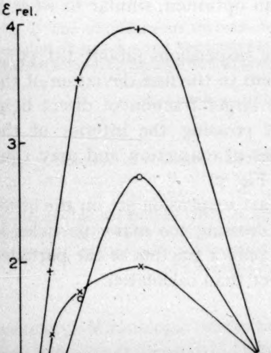

Fig. 3.

Fig. 3. Apparent concentration of the cytochromes *c*,*b* and a_3, calculated from the γ-bands. The yeast suspensions are recorded with 2–8 layers of Munktell no. 3 as reference substance. ×, cytogrome *c*; +, cytogrome *b*; ○, cytogrome a_3.

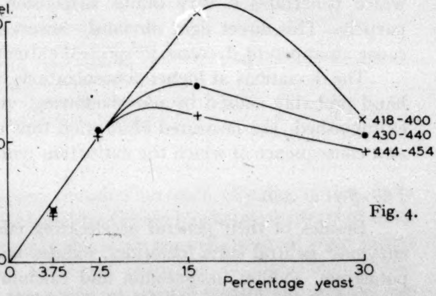

Fig. 4.

Fig. 4. Apparent concentration of the cytochromes *c*,*b* and a_3, calculated from the γ-bands (measured intervals: *c*, 418–400 mμ; *b*, 430–440 mμ; a_3, 444–454 mμ). Difference spectra between two recipients gassed with air and N_2 respectively.

yeast from the cake). The reference beam was turned over into diffuse light by 2–7 layers of colorless filter paper (Munktell No. 3). The figure illustrates the complications brought about by the absorption spectrum of the back-ground. The sloping of the spectrum of denser suspensions is partly caused by increased scattering at decreasing wavelength. A pigment, the transparency of which decreases from blue-green to light red may possibly interfere, too. It is noticed that the vertical position of the cytochrome bands may vary, whereas the shape of the bands is relatively little influenced. Reliable calculations of the apparent concentration of the cytochromes *a*, *b* and *c* may be attained by selection of narrow wave-length intervals, *e.g.* 550–544 (*c*), 562–570 (*b*), and 604–590 (*a*). The corresponding figures are plotted in Fig. 2.

The results show that the apparent concentration of all cytochromes is varying approximately along a straight line up to 10 % concentration of the yeast suspension. At higher concentrations an increasing depression of the α-bands is noticed, primarily regarding cytochromes *c* and *b*, but no reversal of the relative transparency up to 20 % yeast.

Measurements in the γ-region (400–450 mμ) show a considerably more pronounced influence of the concentration. An approximately linear relation between concentration and band height is here obtained only up to 2.5 % (for *c*), 5 % (for *b*) and 5–10 % (for a_3), figures which are in accord with the thesis of decreasing scattering at increasing wave-length (Fig. 3). Somewhat deviating results were, however, obtained from difference spectrograms (the reference was here aerated and the sample gassed with N_2). Fig. 4 shows a linear relation up to 7.5 %, for cytochrome *c* even up to 15 %. Here, too, the apparent band heights are decreasing at high concentrations.

Fig. 5 shows direct measurements of the height of the band at 340 mμ of reduced pyridine nucleotide. A linear relation is here observed up to 5 % yeast suspension. At higher concentrations the curve rapidly reaches an optimum, similar to what is observed in the blue-violet region.

It is often observed that values from very dilute suspensions (about 1–1.35 %) fall somewhat below the straight line running from zero to the first deviation of the curve. These deviations are probably caused by the larger fraction of direct light which penetrates a very dilute suspension without crossing the interior of the particles. This direct light obviously lowers the values of extinction and may thus cause an apparent decrease in spectral extinction (*cf.* Fig. 7).

The deviations at higher concentrations of the yeast suspension are on the other hand probably caused by overshadowing, viz. light crossing too many particles is extinguished. The measured absorption thus refers to only a fraction of the particles as a consequence of which the extinction remains lower than calculated.

The effect of salts

Besides of their general accelerating effect on the activity of the respiratory enzymes[4] neutral salts (chlorides, iodides and bromides, nitrates and sulphates of potassium, sodium, magnesium and calcium) are invariably increasing the transparency of suspensions of washed yeast. Whereas the effect on the enzyme activity starts very rapidly[7] the transparency increases more slowly. This slowly proceeding salt effect is not osmotical, viz. it does not depend on changed dimensions of cells and protoplasm. Experiments with solutions of glucose (0.125–0.500 *M*), which are also osmotically active, revealed no effect on the transparency. The transparency was not affected by salts added to yeast in 5–10 % solutions of ethanol, either.

It is known that living plant cells show an electronegative surface potential, the height of which is regulated by the salt concentration in the medium[9,10]. High salt concentrations are discharging the particles as a consequence of which single cells may show a tendency of clogging together. This would result in increased transparency. Microscopic inspection, however, revealed no such clogging. Other facts are indicating a change in transparency caused by certain biochemical effects of salts.

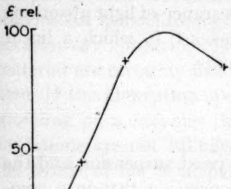

Fig. 5.

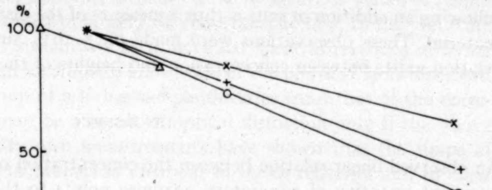

Fig. 6.

Fig. 5. The relation between concentration and apparent extinction at 340 mμ (340–310 mμ) of reduced diphosphopyridine nucleotide.

Fig. 6. The simultaneous increase in transparency of yeast suspension at 540 mμ and the decrease in apparent enzyme concentration following an addition of neutral salts in concentrations of 0.1–0.2 *M*. ×, cytochrome *c*; +, cytochrome *b*; ○, cytochrome *a*; △, diphosphopyridine nucleotide.

Fig. 7. Scheme of repeated reflection (A) and refraction (B) of light in a suspension of spherical cells. It is postulated that the percentage of pure reflected, viz. non-absorbed, light may be of measureable magnitude only in very dilute suspensions (= few cells per unit water) and that refracted, viz. spectrally active, light may be partially extinguished in dense suspensions because of too many crossings.

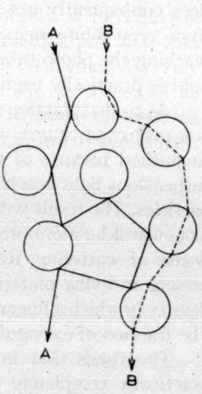

Fig. 7.

As shown in previous communications in this series starved (washed) yeast suffers from a shortage of material (glucose) for rapid reduction of diphosphopyridine nucleotide. As a consequence of that the power of aerobic respiration remains low and it slowly decreases during prolonged aeration. Addition of salts without a corresponding addition of glucose enhances the exhaustion of readily available respiratory fuel and it is likely to assume that now also the structural skeleton, *e.g.* enzymes, are attacked. This would explain why not only the transparency rises but simultaneously also the band heights of all respiratory enzymes are going down more rapidly in the presence of salts. A slowly proceeding destruction of DPN was previously observed also at prolonged aeration in the absence of salts[11] (on other changes brought about by aeration, see [12-14]). This destruction of material accelerates in the presence of salts, but ceases if alcohol or glucose are supplied. The decomposition of the respiratory enzymes may be followed both on reduction in N_2 and on addition of dithionite, thus at largely varying transparency. The decreasing transparency following an addition of salts is thus a measure of the disappearance of light absorbing material. These observations were made on 5–10 % suspensions, in which a linear relation exists between concentration and heights of the bands.

DISCUSSION

An observed linear relation between the concentration of a yeast suspension and the apparent quantity of respiratory enzymes points to the conclusion that in a comparatively large region of dilutions the degree of light scattering remains approximately constant. An upper limit is set when the average distance between the suspended particles attains a critical minimum value. Ingoing light is repeatedly refracted and reflected and crosses a large number of yeast cells before it reaches the photo tube (Fig. 7). Some light is reflected only by the surface of the cells and does consequently not pass through the pigmented interior. The deviating results from very dilute suspensions may be caused by some of this non-absorbed light reaching the photo tube. In more concentrated suspensions this non-absorbed stray-light is practically negligible.

As to that fraction of light that penetrates the cells the number of single crossings is a matter of chance and the probability that some of this light is completely extinguished because of too many crossings grows with the concentration. In dense suspensions light reaching the photo tube has thus crossed only one fraction of the particles. The result will be a seeming decrease of the concentration, and the phenomenon will be more pronounced in the region of short wave-lengths, viz. at a higher degree of scattering. Reliable measurements of the quantity of respiratory pigments present in living material have consequently to be made in that medium region of density in which a linear relation exists between concentration and spectral extinction. The fraction of extinguished light is here negligible.

The thesis that in more dense suspensions a fraction of the ingoing light is practically completely extinguished explains also why spectral bands from turbid solutions are sometimes "flattened" as compared with corresponding bands from clear solutions[3, 16]. The high light absorption in the central part of a band acts similar to a rise in density of the suspension. The "critical minimum" (see above) for a linear relation between molar concentration and extinction is here soon reached as a consequence of which the relative extinction of the peak may be lower than that of the base. This effect grows with increasing density of the sample and/or with decreasing sensitivity of the photo-electric device, may, however, be avoided by using sensitive instruments and keeping the transparency of the sample in the region of linear relation between concentration and extinction, a precaution which was unfortunately neglected by many investigators.

Useful measurements may be made also from more concentrated suspensions, but only if correction factors are introduced. Examples of such corrections are given in a previous communication[2].

As to the question of quantitative determination of respiratory pigments in living material the results teach us that reliable measurements may be made in the region of linear relations between quantity of pigment and spectral extinction. In addition to earlier results it may here be mentioned that under these conditions spectra from samples treated with dithionite, pyridine, alcohol etc. show identical values of hemin enzymes or hemochromogen in spite of largely varying transparency. In the literature occasionally appearing alleged statements as to irregular spectral relations in living material are certainly due to an incomplete knowledge of the physical facts discussed here. If the absorption spectrum of a living sample deviates from that of the corresponding pure enzymes this may be a result of optical distortion only if the above conditions are not fullfilled. My own measurements have shown that the shape of the bands of the principal cytochromes is identical in living material and in clear solutions. Regarding the chlorophyll evidence is accumulating towards the existence of special linkages in the living state[12-15], which change the spectrum, but this question is still under discussion.

Even if a change in the transparency does not appreciably alter measurements of individual band heights the presence of accessory pigments, some of which, *e.g.* quinones and peroxidase, may participate in the general processes of oxidation and reduction, may frequently distort the levels of extinction, notably at recording of broad spectrograms (*cf.* Fig. 1). These disturbances may be avoided provided that the respiratory enzymes are reacting faster than the accessory enzymes. By very rapid recording of the processes of reduction or reoxidation, *e.g.* by means of an oscillographic scanning spectrophotometer, it is thus possible to study the reaction of the cytochromes and flavoprotein apart from that of peroxidase and its substrates[8].

ACKNOWLEDGEMENT

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RÉGULATION DU TAUX DE THYROXINE CELLULAIRE PAR DÉSIODATION ENZYMATIQUE ET SA FIXATION À DES PROTÉINES CELLULAIRES

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(Reçu le 24 octobre, 1959)

SUMMARY

Regulation of the amount of cellular thyroxin by enzymic deiodination and its binding by cell proteins

The adsorption of thyroxin on glass is connected with the ionisation of the phenol group. It is inhibited by the addition to the medium of proteins capable of binding the hormone. There is a parallelism between the binding power of these proteins and the adsorption by the glass. Taking into account these data, which influence the methods applied for the determination of deiodination, some properties of rat muscle and liver deiodinase were specified. The values found for the Michaelis constant indicate that the two enzymes are identical. Their localization in the cell was determined, and also their relations to the cellular proteins capable of binding the thyroxin (TBP_c). The deiodination of thyroxin is inhibited by thyroglobulin, thyrostimulin and TBP_c. In the light of the available data it appears that the deiodination of thyroxin by the tissue constitutes a mechanism controlling the amount of hormone contained in the cell and regulated by a complex cellular distribution of thyroxin and the proteins which concur to bind it (TBP_c, deiodase).

INTRODUCTION

Parmi les problèmes posés par le métabolisme périphérique des hormones thyroïdiennes celui de leur désiodation a donné lieu à de nombreux travaux dont les résultats contradictoires[1] tiennent soit à l'utilisation de techniques analytiques inadéquates, soit à celle de concentrations non physiologiques en hormone. L'iodure minéral est retrouvé par tous les auteurs comme produit de l'action de l'enzyme désiodante et récemment la thyronine a pu être caractérisée dans les milieux d'incubation de coupes de foie avec la thyroxine[2]. Les diverses désiodo-thyroxines, telles que la 3,5,3'-triiodo-L-thyronine, susceptibles de se former au cours de la désiodation de la thyroxine par de nombreux tissus ne doivent pas être considérées comme des

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Les abréviations suivantes seront utilisées: T_4 = L-thyroxine; T_3 = 3,5,3'-triiodo-L-thyronine; ATC = acide trichloracétique; KRP 6.4 = solution ioniquement équilibrée de Krebs-Ringer phosphaté de pH = 6.4; TBP_s = thyroxine-binding protein sérique; TBP_c = thyroxine-binding protein cellulaire.

intermédiaires stables de cette réaction[3-6]; aussi le problème de la signification physiologique de la désiodation demeure-t-il posé.

En ce qui concerne la désiodase, responsable de la dégradation des hormones thyroïdiennes, ses propriétés qualitativement encore assez mal connues, restent à préciser du point de vue quantitatif. L'étude de la localisation morphologique de cette enzyme dans le muscle, le cerveau[4] et le foie[5] a conduit à des résultats contradictoires. Par ailleurs on a pu établir que la thyroxine paraissait unie dans la cellule à une protéine[8-10] dont la spécificité reste à établir (TBP_c) et qu'il devait exister un équilibre de répartition de la T_4 entre la TBP sérique et la TBP cellulaire[8,10].

L'objet de ce travail a été d'étudier sur le plan quantitatif la désiodation de la L-thyroxine par les tissus périphériques (foie et muscle) et d'analyser les relations existantes entre l'enzyme désiodante et la (ou les) protéine(s) fixant la thyroxine.

MATÉRIEL ET MÉTHODES

Des rats mâles (100 à 150 g) ont servi de matériel de départ. Après avoir été sacrifiés par un coup sur la tête et saignés, leurs organes [foie, muscle* (patte postérieure) et rein] sont prélevés immédiatement, placés dans du liquide de Krebs-Ringer phosphaté (KRP) de pH approprié[11] à +2° et coupés en tranches d'environ 0.5 mm d'épaisseur que l'on sèche sur papier filtre et pèse avant leur introduction dans les fioles de Warburg où sera conduite l'incubation.

Le fractionnement des particules cellulaires a été réalisé (0 à +5°) par centrifugation différentielle d'un homogénat de tissu (foie, muscle) dans une solution de saccharose 0.25 *M* selon Hogeboom[12]. Nos recherches ont porté sur l'action désiodante des fractions suivantes: 1. "noyaux" mélangés à des cellules non détruites et des globules rouges, 2. "mitochondries" lavées deux fois, 3. surnageant contenant les microsomes, 4. "microsomes" obtenus par centrifugation du surnageant à 25,000 × *g* pendant 120 min. Chacune des fractions particulaires a été remise en suspension homogène dans le saccharose 0.25 *M* par une rapide homogénéisation. Les essais effectués pour chaque fraction sur des volumes aliquots correspondant à un poids connu de tissu, ont été opérés dans les conditions standard suivantes: incubation dans des fioles de l'appareil de Warburg, à 37° et 60 agitations par min, d'un milieu renfermant au plus 0.050 ml de solution de L-thyroxine marquée en 3',5' par ^{131}I** (solvant: 1,2-propanediol à 50%), l'aliquot porté à 1 ml par addition de saccharose 0.25 *M* et du KRP de pH approprié (ordinairement 6.4) complétant le volume total à 2 ml. Les concentrations en substrat et en tissu seront précisées dans le texte ou en légende des figures. Les mêmes conditions expérimentales ont été mises en oeuvre pour l'étude de l'activité désiodante des coupes, à ceci près que ces dernières baignaient dans un volume total de liquide (KRP) de 1 ml.

Les échantillons chauffés (coupes, homogénats ou fractions cellulaires) ont été portés au bain-marie à 100° pendant 5 min ou des temps appropriés dans des tubes ou fioles munis de réfrigérant à air. Un témoin d'incubation a été réalisé en omettant d'ajouter les coupes ou les fractions cellulaires. Le taux d'iodure contenu dans la solution initiale de L-thyroxine utilisée a été déterminé. Aux erreurs près il a toujours correspondu à la teneur en iodure du témoin d'incubation. Les témoins chauffés ont été exclus pour les raisons exposées plus loin.

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* Le muscle a été choisi parce qu'il représente une part importante du poids de l'individu et qu'il peut être obtenu relativement exsangue. Il possède, en outre, une enzyme désiodante très active[4].

** Synthétisée au laboratoire[13] comme les autres iodothyronines marquées, ou fournie par les laboratoires Abbott (Oak Ridge, Tenn.). Toutes les préparations utilisées contenaient au moins 90% de L-thyroxine. Les concentrations en T_4 supérieures à $1.5 \cdot 10^{-8}$ *M* ont été obtenues en ajoutant aux milieux d'incubation une solution de L-thyroxine non marquée dissoute dans le 1,2-propanediol à 50%.

La mesure de la désiodation a été effectuée à des temps d'incubation compris entre 0 et 180 min (1) par chromatographie sur papier en *n*-butanol–acide acétique–eau (78:5:17) ou amylol tertiaire saturé de NH_4OH $2\,N$; (2) par précipitation de T_4 par l'acide trichloracétique: 0.5 ml du liquide de suspension des essais ou d'homogénat + 0.25 ml plasma de boeuf + 0.15 ml solution IK à 20 mg/ml + 0.9 ml ATC à 20 % dans un tube en verre calibré. La radioactivité totale de ce milieu est mesurée dans un compteur à scintillation à cristal γ creux. On centrifuge, élimine le surnageant et lave deux fois avec 1.8 ml ATC à 10 % contenant 5 mg IK. Le précipité est remis en suspension dans ATC à 10 % et compté. La différence entre les deux mesures donne la quantité d'iodure formée. La détermination de la radioactivité (cristal γ creux) ayant pénétré dans les coupes a été effectuée après lavage de celles-ci à trois reprises par 1.5 ml de KRP 6.4 et dissolution dans KOH à 30 %[8]. Connaissant d'une part la radioactivité initiale mise en oeuvre et, d'autre part, celles des coupes et du liquide dans lequel elles ont baigné, on a par différence la radioactivité adsorbée sur le verre. La désiodation est exprimée en $^{131}I^-$ % de la radioactivité initiale de la [^{131}I]thyroxine ou en mμmole de T_4 désiodée pour un temps donné.

La thyroglobuline de porc[14] et la globine de cheval[15] utilisées étaient homogènes à l'électrophorèse. Le lysozyme provenait de Worthington Lab. Le taux d'azote des fractions cellulaires a été déterminé par micro-Kjeldahl. Tous les réactifs ont été préparés à partir d'eau bidistillée dans un appareil en verre pyrex. Chacune des données expérimentales est la moyenne de deux ou plusieurs essais.

RÉSULTATS

Adsorption de la thyroxine par le verre

Elle varie avec le pH (Fig. 1) et pour une concentration $2.6 \cdot 10^{-8}\,M$ on constate un optimum de fixation vers pH 6.4–6.7. Au-dessous de cette valeur, elle varie peu entre 5 et 6 alors qu'elle décroît très vite au-dessus pour être pratiquement nulle à pH = 9.0. Aux divers pH étudiés, l'adsorption sur le verre est proportionnelle à la concentration (Fig. 2) et d'autant plus forte que le pH est plus bas pour des concentrations supérieures à $1.3 \cdot 10^{-7}\,M$. Au-dessous de cette valeur on retrouve l'optimum de fixation pour pH 6.6, lequel correspond au pK de la fonction phénolique de la T_4[16].

L'adsorption sur le verre de la T_4 peut être inhibée par l'addition de sérum ou de plasma (Fig. 3) jusqu'à une valeur limite correspondant à une dilution au 1/50ème, et pour une concentration $1.3 \cdot 10^{-8}\,M$ en T_4. L'augmentation de la concentration en plasma ne modifie plus l'adsorption de la T_4 par le verre, laquelle est alors minimum. Toutes les protéines capables de fixer la T_4 sont également très actives (thyroglobuline, TBP_c, thyréostimuline (Tableau I). Le siliconage des fioles (Rhodorsil, Rhône Poulenc) inhibe la fixation sur le verre d'environ 50 % pour 10^{-8} à $10^{-7}\,M$ en T_4.

Sur le plan pratique, la mesure de l'activité désiodante de milieux pauvres en protéines pourra être considérablement perturbée par l'adsorption de T_4 sur le verre: l'iodure n'étant pas adsorbé, (Tableau II) la valeur $^{131}I^-$ % sera augmentée d'une valeur proportionnelle à la T_4 adsorbée. L'emploi de la méthode à l'acide trichloracétique permet de faire le bilan de la radioactivité après incubation et par là d'effectuer les corrections dues à l'adsorption. Les données du Tableau III indiquent les éléments numériques du calcul.

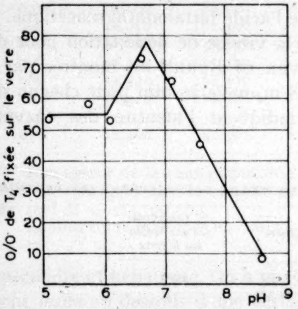

Fig. 1. Variation de la quantité de L-thyroxine fixée sur le verre en fonction du pH; concentration en T_4: $2.6 \cdot 10^{-8}$ *M*. (Composition des tampons et conditions expérimentales, voir Fig. 2).

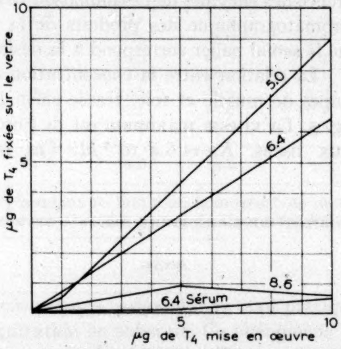

Fig. 2. Fixation de la thyroxine sur le verre en fonction de la quantité mise en oeuvre à différents pH: 5.0 (acétoacétique); 6.4 (phosphates); 8.6 (véronal); 6.4 + sérum (0.15 ml de sérum ajouté au milieu d'incubation). Volume total 1 ml — 60 min d'incubation à 37°.

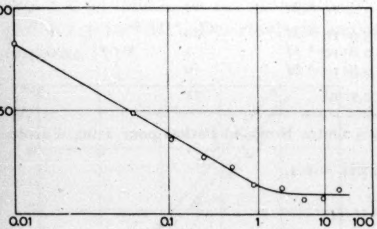

Fig. 3. Inhibition de la fixation de T_4 sur le verre par le plasma (boeuf); mêmes conditions expérimentales que dans Fig. 2. 1 ml plasma contenait 80 mg de protéines totales.

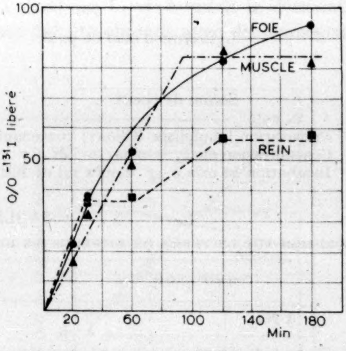

Fig. 4. Cinétique de la désiodation de L-thyroxine par des coupes de foie, muscle et rein de rat. 150 mg de coupes dans 1 ml de KRP 6.4. T_4: $6.4 \cdot 10^{-8}$ *M*.

Le Tableau II montre que les autres iodothyronines sont beaucoup moins adsorbées, l'iodure et les iodotyrosines ne l'étant pas.

Désiodation de la L-thyroxine par le foie et le muscle de rat

La cinétique de la désiodation de T_4 par divers tissus de rat, étudiée par chromatographie ou par la méthode de précipitation (ATC), montre que les vitesses initiales de dégradation sont plus élevées pour le foie et le rein (1.2 à 1.4 $m\mu$moles/10 min/150 mg coupes) que pour le muscle (0.6 à 0.8 $m\mu$mole/10 min/150 mg coupes) pour T_4 $6.4\cdot10^{-8}\,M$ (Fig. 4). La désiodation maximum est atteinte dans le même temps (120 min) pour foie et muscle alors qu'elle n'est que de 60 % pour le rein en 180 min. Les deux paliers de la courbe de désiodation par ce dernier tissu traduisent l'interférence des enzymes de désamination très abondants dans le cortex rénal[17, 18]. L'étude chromatographique des produits de la réaction à divers temps a permis d'établir que le seond palier correspond à la désiodation de l'acide tétraiodothyroacétique.

La relation entre la concentration en T_4 et la vitesse de désiodation pour des coupes de muscle et foie, tracée selon Lineweaver et Burk[19] est montrée par la Fig. 5. La vitesse maximum est de l'ordre de 0.8 $m\mu$mole/20 min pour chacun des deux tissus. $Ks = 6.4\cdot10^{-7}\,M$. Ces résultats indiquent l'identité des enzymes

TABLEAU I

INHIBITION DE L'ADSORPTION DE LA L-THYROXINE SUR LE VERRE PAR DIFFÉRENTES PROTÉINES

Protéine	*Concentration*	*% Inhibition de l'adsorption sur le verre*
Thyroglobuline de porc	$1.6\cdot10^{-9}\,M$	100
	$1.6\cdot10^{-10}\,M$	19
Thyréostimuline*	1.25 unités R.P.	91
	0.25	42
	0.025	27
	0.0025	0
Globine de cheval	$3.8\cdot10^{-4}\,M$	92
	$1.9\cdot10^{-4}\,M$	91
	$3.9\cdot10^{-5}\,M$	28
	$3.9\cdot10^{-6}\,M$	0
Lysozyme d'oeuf de poule	$3.9\cdot10^{-4}\,M$	20
	$0.8\cdot10^{-4}\,M$	1
	$0.8\cdot10^{-5}\,M$	0
Sérum de boeuf	0.1 ml	71

* Préparation lyophilisée (Choay) contenant 2.5 unités Rowland-Parkes pour 2 mg d'azote.
Concentration en $T_4 = 0.64\cdot10^{-7}\,M$.
Incubation 60 min à 37° dans 1 ml de KRP, pH = 6.4.

TABLEAU II

ADSORPTION SUR LE VERRE DE DIFFÉRENTES IODOTYROSINES, IODOTHYRONINES ET DE L'IODURE

Composés étudiés	*Concentration M*	*% Adsorbé sur le verre*
I Na	traceur	0**
	$0.3\cdot10^{-8}$ à $3\cdot10^{-6}$	0
3-iodo-L-tyrosine et 3,5-diiodo-L-tyrosine*	$1.2\cdot10^{-7}$ à $3\cdot10^{-6}$	0
L-thyroxine	$3.5\cdot10^{-8}$	73.1
3,5,3′-triiodo-L-thyronine	$3.5\cdot10^{-8}$	40.0
3,3′,5′-triiodo-L-thyronine	$3.0\cdot10^{-8}$	52.0
3,3′-diiodo-L-thyronine	$3.8\cdot10^{-8}$	30.4
3′,5′-diiodo-L-thyronine	$3.8\cdot10^{-8}$	1.0
N-acetyl-L-thyroxine	$3.2\cdot10^{-8}$	16.5

Chaque fiole contenait le corps étudié amené dans un volume égal ou inférieur à 0.050 ml et la quantité de KRP 6.4 nécessaire pour amener le volume à 1 ml; 60 min d'incubation à 37°.

* Les iodotyrosines sont marquées par ^{131}I en 3 et/ou 5, les iodothyronines en 3′ et/ou 5′.

** Chaque valeur correspond à la moyenne de 4 à 5 déterminations.

TABLEAU III

ACTIVITÉ DÉSIODANTE DU SURNAGEANT D'UN HOMOGÉNAT DE MUSCLE DE RAT

*Volume du surnageant (ml)**	*Radioactivité de ^{131}iodure*	*Radioactivité totale du milieu d'incubation*	*Radioactivité adsorbée par le verre*	*% Radioactivité fixée sur le verre*	*% Iodure libéré non corrigé*	*% Iodure corrigé de l'adsorption sur le verre*	*% Iodure corrigé de celui contenu dans le témoin*
0.02	15,800	60,040	153,060	71.8	26.2	7.4	1.6
0.05	19,100	88,520	124,580	58.5	21.5	8.9	3.2
0.1	48,010	162,450	50,640	33.8	29.5	22.5	16.7
0.2	40,180	148,650	64,440	30.2	27.0	18.9	13.1
0.5	38,300	157,430	55,670	26.2	21.6	18.0	12.2
0.8	33,930	168,300	44,790	21.1	20.1	15.9	10.1
1.0	38,510	176,310	36,790	17.2	21.8	16.6	11.8
0	12,460	41,390	171,700	80.7	30.6	5.8	

* Surnageant de la centrifugation à 24,000 × *g* pendant 10 min d'un homogénat de muscle de rat au 1/10 (p/v) dans le saccharose 0.25 *M*. 1 ml correspond à 33 mg de tissu frais. ^{131}I-T_4: $0.87 \cdot 10^{-8}$ *M* = 213,100 c/m.

Radioactivité exprimée en coups/min.

musculaire et hépatique. On a par ailleurs constaté que la désiodation était pratiquement nulle au-dessous d'une certaine concentration en substrat. Ce phénomène est bien mis en évidence lorsqu'on étudie la désiodation par des quantités croissantes de coupes pour des concentrations données en T_4 (Fig. 6). Pour de faibles concentrations en T_4 on constate un effet inhibiteur de la désiodation très marqué, et pour $1.3 \cdot 10^{-9}$ *M* la quantité de T_4 désiodée décroît régulièrement lorsqu'on augmente le poids de coupes de tissu. Si l'on extrapole cette droite au point d'abscisse 0, on trouve que 1 g de muscle de rat protège 0.0008 à 0.001 μg T_4 vis-à-vis de la désiodation (0.8 à $1.3 \cdot 10^{-9}$ *M*)*. Des résultats analogues ont été obtenus avec des homogénats (Tableau IV)[23].

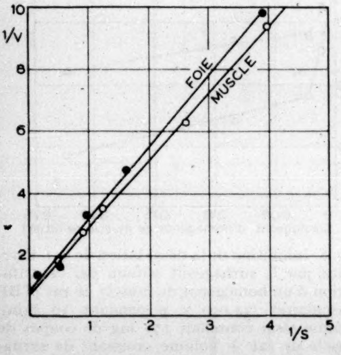

Fig. 5. Relation entre la concentration en thyroxine et la vitesse de désiodation; 150 mg de coupes dans un volume total de 1 ml (KRP 6.4), 20 min d'incubation à 37°.

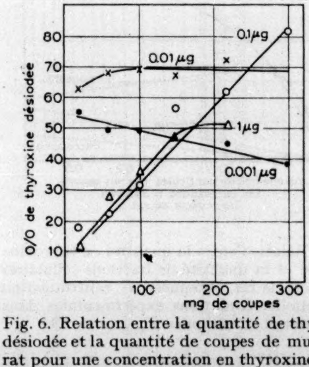

Fig. 6. Relation entre la quantité de thyroxine désiodée et la quantité de coupes de muscle de rat pour une concentration en thyroxine variable (0.001 à 1 μg). Volume total des milieux d'incubation 1 ml, poursuivie à 37° pendant 60 min.

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* Valeurs extrêmes pour un lot homogène de rats. Une assez grande variabilité a été cependant constatée selon la provenance et l'état physiologique de ces animaux.

TABLEAU IV

ACTIVITÉ DÉSIODANTE D'HOMOGÉNATS DE MUSCLE ET DE FOIE DE RAT EN FONCTION DE LA QUANTITÉ DE CEUX-CI

Homogenat	*Quantité de tissu correspondant (mg)*	*Concentration en T_4 M*	*% de ^{131}I libéré*		*% de Radioactivité fixée sur le verre*	
			frais	*chauffé*	*frais*	*chauffé*
Muscle	5	$0.87 \cdot 10^{-8}$	32.0	40.1	15.9	27.9
	25	$0.87 \cdot 10^{-8}$	23.5	30.0	14.0	33.2
	50	$0.87 \cdot 10^{-8}$	12.1	19.0	13.6	38.6
Foie	10	$1.7 \cdot 10^{-8}$	27.7	28.9	6.4	7.3
	25	$1.7 \cdot 10^{-8}$	24.1	27.2	5.3	9.1
	50	$1.7 \cdot 10^{-8}$	18.8	26.8	4.1	12.2
	100	$1.7 \cdot 10^{-8}$	4.3	25.7	3.2	15.4

Temps d'incubation: 20 min à 37° dans KRP 6.4.

L'existence d'une TBP cellulaire[8–10] permet d'expliquer cet effet de protection vis-à-vis de la désiodation en postulant que la désiodase, active sur la T_4 libre, ne l'est pas sur T_4 combinée avec une protéine, ce que démontrent les expériences suivantes. Les fractions cellulaires isolées par centrifugation différentielle ont été essayées dans les mêmes conditions. Le Tableau V et la Fig. 7 montrent que l'activité désiodante est située dans le surnageant du foie alors qu'on la trouve dans les mitochondries et le surnageant du muscle. L'activité protectrice est localisée dans la fraction soluble des deux tissus: son addition à des coupes, inhibe leur activité désiodante, en même temps que le % de radioactivité dans les coupes diminue (Fig. 8, Tableau V). Le sérum sanguin, la thyroglobuline, la thyréostimuline* et à

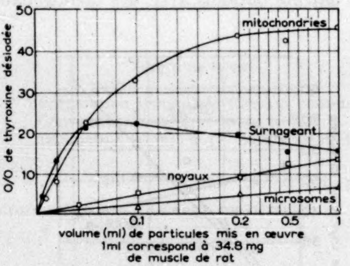

Fig. 7. Relation entre la quantité de thyroxine désiodée et la quantité de fractions cellulaires de muscle de rat, obtenues par centrifugation différentielle. Conditions expérimentales dans matériel et méthodes. T_4: $4.9 \cdot 10^{-8}$ *M*.

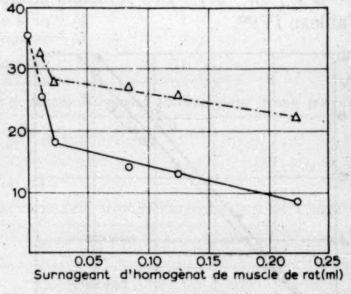

Fig. 8. Inhibition de la désiodation de la L-thyroxine par le surnageant obtenu par centrifugation d'un homogénat de muscle de rat (TBP musculaire) (24,000 × *g* pendant 10 min). Chaque essai contenait 150 mg de coupes de muscle de rat + volume croissant de surnageant + KRP 6.4 pour un volume total de 1 ml; 1 ml de la solution de surnageant correspondant à 2 g de tissu. Ordonnées: courbe en trait plein, % de T_4 désiodée; courbe en pointillé, % de la radioactivité totale ayant pénétré dans les coupes. Le point d'abscisse O correspond au témoin sans surnageant.

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[*] Préparation contenant 0.5 Unités Rowland-Parkes/mg, souillée d'autres protéines d'origine hypophysaire.

TABLEAU V

Préparation tissulaire	*Muscle*					*Foie*				
	% de ${}^{131}I^-$ libéré			*% de Radioactivité fixée sur le verre*		*% de ${}^{131}I^-$ libéré*			*% de Radioactivité fixée sur le verre*	
	Frais	*A.S.** relative*	*Chauffé**	*Frais*	*Chauffé**	*Frais*	*A.S.** relative*	*Chauffé**	*Frais*	*Chauffé**
Homogénat total	21.2	100	26.1	14.2	31.5	28.7	100	28.9	6.4	7.3
Fraction "noyaux"	13.5	15	0.0	23.6	22.6	10.1	60	2.1	18.2	17.3
Fraction mitochondries	*45.2*	2200	4.9	*16.9*	*58.2*	12.7	170	3.6	17.4	18.2
Surnageant après 10 min à 24,000 × *g*	15.3	170	2.2	*6.4*	*21.2*	42.5		30.4	10.7	16.5
Fraction microsomes	7.2	—	3.7	*8.5*	*63.2*	20.1	820	4.0	*5.7*	*28.4*
Fraction soluble	0.0	—	0.0	*4.3*	*59.7*	25.4	300	12.5	*14.0*	*35.4*
Fraction mitochondries + fraction soluble	*22.1*	—	2.1	*3.0*	—	—	—	—	—	—

* 5 min à 100°.

** Activité spécifique relative (${}^{131}I$-%/mg d'azote/20 min).

Conditions expérimentales: chaque fiole contenait l'équivalent de 35 mg (muscle) et de 7 mg (foie) de tissu de rat apporté dans 1 ml de saccharose 0.25 *M*, 0.05 μg de T_4 (muscle) et 0.027 μg (foie); volume total complété à 2 ml avec KRP pH 6.4.

un moindre degré la globine ont également une forte action inhibitrice (Tableau VI). Dans le cas de la thyréostimuline, cependant, le % de radioactivité ayant pénétré dans les coupes, augmente avec la quantité de thyréostimuline ajoutée.

TABLEAU VI

INHIBITION DE LA DÉSIODATION DE LA L-THYROXINE (COUPES DE MUSCLE DE RAT) PAR DIFFÉRENTES PROTÉINES

Protéine ajoutée	*Concentration*	% ^{131}*Iodure libéré*	% *Inhibition de la désiodation*	% *Radioactivité ayant pénétré dans les coupes*
Thyroglobuline (porc)	$0.16 \cdot 10^{-8}\,M$	78.8	0	26.0
	$0.79 \cdot 10^{-8}\,M$	72.9	7.8	29.7
	$1.5 \cdot 10^{-8}\,M$	48.1	39.1	40.5
	$3.0 \cdot 10^{-8}\,M$	28.5	63.9	44.3
Plasma de boeuf	0.1 ml	22.7	56.8	34.1
Thyréostimuline*	0.025 URP	76.1	0	47.6
	0.12	72.3	4.8	51.2
	0.25	68.8	9.5	59.7
	0.5	45.9	39.5	69.1
	1.0	38.3	49.6	69.8
Globine (cheval)	$5.8 \cdot 10^{-7}\,M$	46.3	37.3	48.1
Sérumalbumine (boeuf)	$1.3 \cdot 10^{-7}\,M$	80.1	0	14.5
Sans	—	79.0–74.2	—	44.5

* Préparation lyophilisée (Choay) contenant 2.5 unités Rowland-Parkes pour 2 mg d'azote total.

Action du chauffage sur la désiodation

Le chauffage des *coupes* de foie ou de muscle pendant 5 min à 100° diminue légèrement le % de désiodation pour des concentrations 1.3 à $2.6 \cdot 10^{-8}\,M$ en T_4; il augmente celui des homogénats, l'effet étant d'autant plus marqué que le rapport Homogénat/T_4 est plus élevé. Par contre, il inhibe *complètement* l'activité désiodante des fractions cellulaires isolées, à l'exception de la fraction soluble du foie qui n'est que partiellement inhibée (Tableau V) dans ces conditions.

L'adsorption de T_4 sur le verre augmente notablement à la suite du chauffage des coupes, des homogénats, des fractions mitochondriale ou soluble du muscle et de la fraction soluble du foie (Tableau V). Cet effet peut s'interpréter comme traduisant la thermolabilité des protéines cellulaires unissant le T_4 que l'on trouve aussi bien dans la fraction soluble que dans les microsomes. Les mitochondries du muscle se comportent pareillement. La fixation de la T_4 sur le verre ne varie pas, pour les noyaux, avec le chauffage.

DISCUSSION

Comme l'ont déjà noté FREINKEL *et al.*[8] l'importance de l'adsorption de la thyroxine par le verre à partir de ses solutions salines est un facteur dont il faut tenir compte lorsqu'on étudie le métabolisme *in vitro* de l'hormone, dans des milieux pauvres en protéines capables de la fixer. Le sérum inhibe fortement cette adsorption ainsi que la thyroglobine, la thyréostimuline et les milieux contenant la TBP cellulaire. Les autres iodothyronines sont beaucoup moins fixées par le verre. L'étude de l'interaction de ces dernières avec la TBP_s a mis en évidence l'ionisation de l'hydroxyle phénolique comme facteur indispensable pour l'union avec la protéine[20]; il en est de même de l'effet de "stabilisation" des TBP vis-à-vis de l'instabilité transitoire des iodothyronines par dilution avec un tampon[21]. Il existe un parallélisme frappant entre ces deux propriétés des protéines capables de se combiner à la thyroxine et l'inhibition qu'elles exercent sur la fixation de la thyroxine par le verre. Toutes trois sont liées à l'ionisation de la fonction phénolique de cette dernière et si le mécanisme de telles interactions demeure inconnu il met l'accent sur la réactivité de la thyroxine dans les milieux biologiques et sur l'importance physiologique éventuelle de celles-ci.

L'activité désiodante du foie et du rein de rat est plus élevée que celle du muscle, la valeur trouvée pour la constante de Michaëlis montrant par ailleurs l'identité des enzymes musculaire et hépatique. La courbe $1/V$ en fonction de $1/S$ n'est pas une droite dans toute la gamme des concentrations utilisées et s'infléchit vers le haut pour les faibles concentrations en substrat (entre $1.2 \cdot 10^{-8}\,M$ et $1.2 \cdot 10^{-9}\,M$). On obtient de telles courbes dans le cas d'un inhibiteur qui s'unit au substrat en l'absence ou en présence de l'enzyme[21] ce qui se traduit par une courbe non linéaire difficile à distinguer de celle correspondant à une inhibition compétitive. L'existence d'une protéine cellulaire fixant la T_4[8–10], située principalement dans la fraction soluble du cytoplasme[10], permet d'interpréter les résultats des Figs. 6 et 7 avec $K_{s.\mathrm{TBP_c}} < K_{s,\mathrm{E}}$.* et de rendre compte du phénomène observé. La complexité du matériel utilisé (coupes ou homogénats) ne permet pas une évaluation précise de $K_{s.\mathrm{TBP_c}}$. Cependant, l'extrapolation des courbes 0.001 μg de la Fig. 6 permettent d'évaluer la capacité de fixation de T_4 par la TBP_c *libre* musculaire, en présence de désiodase; elle est de l'ordre de 0.0008 à 0.001 μg/g de tissu (soit 1 à $1.3 \cdot 10^{-9}\,M$) et correspond à la quantité de T_4 protégée vis-à-vis de la désiodation.

L'addition de la fraction soluble d'un homogénat de muscle inhibe, proportionnellement à la quantité ajoutée, la désiodation de T_4 par les coupes de muscle. Le sérum sanguin, la thyréostimuline et la thyroglobuline ont également un pouvoir inhibiteur intense, alors que la sérumalbumine et la globine sont beaucoup moins actives. Une étude mettant en jeu l'électrophorèse et la méthode de précipitation par l'acide trichloracétique a par ailleurs montré qu'il existe un rapport direct entre l'activité inhibitrice de ces protéines vis-à-vis de la désiodation de la T_4 et leur affinité pour l'hormone[23]. Il existe donc une compétition de la désiodase et de la TBP_c pour la T_4 dans les tissus, une quantité déterminée de celle-ci se trouvant protégée vis-à-vis de la désiodation. Étant donné qu'il n'a pas été établi jusqu'à présent que la désiodation de la T_4 conduise à des corps physiologiquement plus actifs[1] ou présentant les caractères d'une hormone active**[24], on doit donc considérer la désiodation de la T_4 par les tissus périphériques comme un mécanisme de régulation cellulaire du taux de thyroxine libre. Dès lors, en plus d'un équilibre de distribution de T_4 entre la TBP sérique et la TBP cellulaire, un nouvel équilibre entre la TBP cellulaire et la désiodase doit être envisagé. La purification en cours de ces protéines permettra de préciser la cinétique de ces réactions. Il est intéressant de noter que l'action inhibitrice de la thyréostimuline sur la désiodation de la thyroxine va de pair avec son rôle activateur de la pénétration de l'hormone dans les coupes de tissu *in vitro*. Ces faits traduisent une possibilité d'action périphérique de la stimuline sur le métabolisme cellulaire périphérique de l'hormone.

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* $\dfrac{[\mathrm{TBP_c}]\,[\mathrm{T_4}]}{[\mathrm{TBP_c} \cdot \mathrm{T_4}]} = K_{s\cdot\mathrm{TBP_c}}$ $\quad \dfrac{[\mathrm{E}]\,[\mathrm{S}]}{[\mathrm{E} \cdot \mathrm{S}]} = \mathrm{K}_{s\cdot\mathrm{E}}$ avec $T_4 = S$ et Désiodase $= E$.

** Toutes les iodothyronines pouvant provenir de la désiodation de la thyroxine agissent comme elle après un temps de latence.

La présence de l'enzyme désiodante dans la fraction soluble du foie et du muscle est en accord avec les résultats de TATA[4]. Cependant, contrairement à cet auteur, nous avons constaté que les mitochondries du muscle contiennent la plus grande part de l'activité désiodante. Cette divergence pourrait être expliquée par des différences dans les méthodes de préparation des homogénats et de mesure de la désiodation. L'activité désiodante des fractions isolées par centrifugation différentielle est totalement inhibée par un chauffage de 5 min à 100°, alors que ce traitement exalte l'activité des coupes et des homogénats. Par ailleurs, le pouvoir inhibiteur de la fixation de T_4 sur le verre des fractions chauffées (fraction soluble et fraction microsomes) est considérablement diminué (Tableau V). Ces faits traduisent la thermolabilité plus importante de la TBP_c par rapport à la désiodase. L'activation par chauffage du pouvoir désiodant des homogénats paraît donc être due à la dénaturation partielle de la TBP_c qui libérerait l'enzyme, beaucoup plus thermostable, d'un inhibiteur concourant avec elle pour s'unir à la thyroxine et se trouverait en association étroite avec elle dans la cellule. L'inactivation totale des fractions cellulaires isolées est cependant en contradiction partielle avec cette interprétation. On peut envisager qu'elle résulte d'un type d'union particulier entre la TBP_c et la désiodase dans la cellule et les homogénats, lequel serait détruit au cours des manipulations de centrifugation différentielle.

Cette situation cellulaire doit par ailleurs varier avec les tissus étudiés, l'activité désiodante des homogénats de foie[25,3] et de coupes de rein[26] n'étant pas affectée par un chauffage de 30 min et de 10 min à 100° respectivement, alors qu'il semble ne pas en être de même pour le muscle[10]. On notera par ailleurs (Tableau V) que la répartition de l'activité désiodante dans les fractions cellulaires du foie est beaucoup moins nette qu'elle ne l'est dans le muscle. Les faits exposés ici semblent donc indiquer que la thermostabilité de l'activité désiodante est liée à son union physiologique avec certains constituants cellulaires dont la TBP_c représente un élément important.

RÉSUMÉ

L'adsorption de la thyroxine sur le verre est liée à l'ionisation de la fonction phénolique. Elle est inhibée par l'addition au milieu de protéines capables de fixer l'hormone. Un parallèlisme existe entre le pouvoir de fixation de ces protéines et l'adsorption par le verre. En fonction de ces données qui influent sur les méthodes de mesure de la désiodation, certaines propriétés de la désiodase du muscle et du foie de rat ont été précisées. Les valeurs trouvées pour la constante de Michaëlis montrent l'identité des deux enzymes. Leur localisation cellulaire a été précisée ainsi que leurs rapports avec les protéines cellulaires capables de fixer la thyroxine (TBP_c). La désiodation de cette dernière est inhibée par la thyroglobuline, la thyréostimuline et la TBP_c. En fonction des données actuelles, la désiodation de la thyroxine par les

tissus, constitue un mécanisme de régulation du taux cellulaire de l'hormone conditionné par une répartition cellulaire complexe entre celle-ci et les protéines concourant pour l'unir (TBP$_c$, désiodase).

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THE INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO A SPECIFIC LIPID FRACTION AND INTO ADENINE NUCLEOTIDES OF MITOCHONDRIA DURING OXIDATIVE PHOSPHORYLATION

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SUMMARY

A method for the extraction of mitochondrial lipid using acetone to precipitate the mitochondrial lipoprotein is reported. This procedure gave rapid destruction of enzymic activity and high phosphatide recoveries under mild conditions and was used to simultaneously follow the incorporation of [^{32}P]orthophosphate into the adenine nucleotides and the lipid components of mitochondria during oxidative phosphorylation.

At 10° the incorporation of [^{32}P]orthophosphate into ATP proceeded rapidly and linearly during the initial minutes of reaction, but leveled off after 10 to 15 min. ADP behaved similarly but the specific activity remained about half that of the ATP.

The incorporation into the lipids at 10° occurred almost completely into a very minor fraction; however, this incorporation was not detectable for the first 10 min of reaction, after which it increased rapidly. The conclusion was made that unless this lipid fraction contains a minor but highly active component, it cannot be the precursor of the phosphate moiety of ATP and therefore cannot act as an intermediate in the phosphate esterification reactions during oxidative phosphorylation.

INTRODUCTION

The localization of high concentrations of lipids in mitochondria[1–5], and their association with the insoluble oxidative enzymes[6,7] and sub-mitochondrial particles capable of carrying out oxidative phosphorylation[8–10], have suggested an important role for these compounds in mitochondrial function. However, there is as yet no clear evidence to indicate whether such a role might be as a specific reactant in oxidative phosphorylation or merely as a component in the structural organization.

Abbreviations: TCA, trichloroacetic acid; AMP, adenosine 5′-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DNP, 2,4-dinitrophenol.

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With the development of paper and column chromatographic methods for phosphatide analysis, a high degree of resolution of phospholipid preparations became possible. This increased resolution has revealed a number of minor constituents hitherto unobserved[11, 12] and raises the question of a specific role for these phosphatides.

Studies with isolated *in vitro* systems[3, 13] have shown that unidentified acidic glycerolphosphatides, which are found nearly exclusively in mitochondria, rapidly incorporate [^{32}P]orthophosphate during oxidative phosphorylation. During such incubation the common phosphatides showed very little incorporation of radioactivity. In this study the incorporation of [^{32}P]orthophosphate into the individual phosphatide components of mitochondria was determined during oxidative phosphorylation and compared with the incorporation into ATP. These studies indicate that the phosphatides do not incorporate [^{32}P]orthophosphate at a rate fast enough to suggest their direct participation as intermediates in oxidative phosphorylation.

EXPERIMENTAL

Methods and reagents

Mitochondria were prepared by the method of DOUNCE *et al.*[14] modified by the omission of the citric acid. Paper chromatographic analysis of the phosphatides was carried out by procedures previously described[15]. Paper chromatographic analysis of the adenine nucleotides was carried out by modifications of the procedures of EGGLESTON AND HEMS[16] and KREBS AND HEMS[17]. The methods for lipid detection have been described previously[11, 18]. Autoradiograms were prepared on Kodak no-screen X-ray film and required 3 to 4 weeks' exposure. The radioactivity was determined on the paper spots which were cut from the chromatograms. Phosphorus determinations were made by a modification of the method of CHEN *et al.*[19] or by the method of HARRIS AND POPAT[20].

Preparation of lipid extracts

Preliminary studies on the preparation of lipid extracts from TCA precipitates of mitochondria revealed that a considerable loss of both lipid and lipid phosphorus was incurred. Paper chromatographic analysis of such extracts demonstrated some degradation of phosphatide and the appearance of a lipid material whose chromatographic behavior was very similar to lysophosphatides.

From the results of these experiments it was felt that a non-acidic precipitant would cause less lipid degradation, particularly of a possible labile phosphate intermediate. Acetone was chosen on the basis of its use as a phosphatide precipitant and its high solubility in water. Phosphatides show maximum insolubility in acetone when the acetone is anhydrous. Such conditions would not exist during the mitochondria precipitation. It was felt, however, that the phosphatides of mitochondria might also be relatively insoluble in acetone-water mixtures containing acetone in concentrations of 50 % or less. Various concentrations of acetone were therefore tested at 0° and 45° by pipetting a suspension of mitochondria into the appropriate volume of acetone. It was found that concentrations between 40 and 50 % acetone gave nearly complete precipitation of mitochondrial protein. The effect of temperature did not appear to be appreciable on the extent of protein precipitation, but lower temperatures (0° *versus* 45°) retarded considerably the rate of protein flocculation.

Lipid extracts were prepared from washed precipitates of mitochondria by heating at 60° for 4 min with chloroform–methanol (1:1). This extract was dried under nitrogen in vacuo and the residue re-extracted with warm chloroform–petroleum ether–methanol (5:5:1). With mitochondria precipitated by 40 or 50 % acetone at 45° and washed once with 40 % acetone, the recovery of lipid phosphorus was 80–90 % when compared with the recovery obtained by the direct extraction of KCl-washed mitochondria (Table I). The lipid (dry wt.) usually varied between 6.0 and 6.5 mg/ml of mitochondrial preparation. Extraction of the combined acetone supernatant fluids and wash solutions with chloroform–petroleum ether (1:1) yielded less than 10 % of the lipid phosphorus and about 15–20 % of the lipid (dry wt).

TABLE I

THE RECOVERY OF LIPID FROM TRICHLOROACETIC ACID AND ACETONE PRECIPITATES OF RAT-LIVER MITOCHONDRIA

Expt.	*Precipitant**	*Precipitation temperature*	*Lipid phosphorus*	
			mg/ml mitochondria	*% recovery*
I	5 % TCA	0°	0.36	64
	Control**	0°	0.57	100
II	40 % acetone	0°	0.30	72
	50 % acetone	0°	0.34	81
	40 % acetone	45°	0.37	88
	50 % acetone	45°	0.35	83
	Control**	45°	0.42	100

* Protein loss was determined by observing the precipitate formed on addition of one half volume of 20 % TCA to the supernatant fluid at 50°.

** Lipid P recovery from the direct extraction of KCl washed mitochondria served as the control.

Fig. 1 shows a chromatographic comparison of the phosphatides obtained by direct extraction of mitochondria, the extraction of an acetone precipitate, and extraction of the combined acetone supernatant fluid and wash solution. No qualitative differences were detected chromatographically between the two methods. Chromatography of the lipid extracted from the combined acetone supernatant fluid and wash solution demonstrated small amounts of lecithin, phosphatidyl ethanolamine, and inositol phosphatide and only trace amounts of the other phosphatides. It is noteworthy that in a preparation of mitochondria incubated with [^{32}P]phosphate, the combined acetone supernatant solution and wash solution contained a labeled material which behaved on chromatograms like a lysophosphatide. This was the only labelled phosphatide material detected on autoradiograms in the extracts of these solutions. Moreover, the phosphatide was not detectable in the extracts of the acetone precipitates of mitochondria. The method for lipid extraction may therefore lead to losses of certain lipids, in particular water soluble lipids like the lysophosphatides.

Incorporation of [^{32}P]orthophosphate into the phosphatides of mitochondria

When mitochondria were incubated for 20 min with [^{32}P]orthophosphate in the presence of appropriate substrates, a lipid extract was obtained which was radioactive. Chromatography and subsequent autoradiography of the extracted lipids revealed that the radioactivity was almost exclusively in 3 or 4 spots (Fig. 1-D). In agreement with earlier work[3, 13] extremely little or no activity was detected in the common phosphatide components such as lecithin, phosphatidyl serine, phosphatidyl ethanolamine, or sphingomyelin.

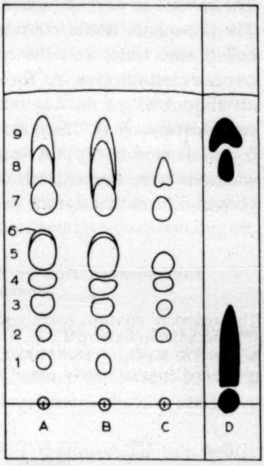

Fig. 1. Paper chromatograms and autoradiogram of total lipid extracts of rat liver mitochondria and acetone precipitates of mitochondria. A, the lipids were prepared by direct extraction of mitochondria. B, the lipids were prepared by extraction of acetone precipitates of mitochondria. C, the lipids were prepared by extraction of the acetone supernatant fluid and wash solution. D, tracing of an autoradiogram of ^{32}P labeled mitochondrial lipids. The lipids in this case were prepared by direct extraction of the mitochondria. Chromatography was carried out on silicic acid impregnated paper as described previously[15]. In A, B, and C the lipid spots were detected by staining with Rhodamine 6 G. In D the spots were detected by exposure of X-ray film. The identification of spots is as follows[15]: spots 1 and 2, unidentified; spot 3, inositol phosphatide; spot 4, sphingomyelin; spot 5, lecithin; spot 6, phosphatidyl serine; spot 7, phosphatidylethanol- amine; spots 8 and 9, polyglycerolphosphatides.

One of the spots observed on the autoradiograms was located near the origin and corresponded to none of the lipid spots detected with the dye, Rhodamine G. These origin components are believed to be traces of inorganic phosphate, ATP, and other water soluble phosphates.

A second faint elongated spot (R_F value of about 0.20–0.30) is believed to be due to traces of lysophosphatide. It was found in lipid extracts prepared directly on mitochondria. However, with acetone precipitation it was not found in the extracts of the mitochondrial precipitate, but rather occurred in extracts of the acetone supernatant fluid and washes.

The major portion of the radioactivity was found in spots 8 and 9 (Fig. 1). These components are believed to be polyglycerolphosphatides[3]. The autoradiograms sometimes revealed a double spot, but often the radioactivity was concentrated along the leading edge of spot No. 9, suggesting a material of rather low concentration but with a high specific activity. In the following rate studies the total values for spots No. 8 and No. 9 were used so as to minimize variation in specific activity due to this unequal distribution.

The incorporation of [^{32}P]orthophosphate into this fast moving phosphatide component was very sensitive to uncoupling of oxidative phosphorylation by 10^{-4} M DNP. KCN in concentrations of 10^{-3} M, inhibited both respiration and [^{32}P]orthophosphate incorporation into ATP, but gave a less pronounced inhibitory effect on the incorporation into this lipid than did DNP.

Rate of incorporation of [^{32}P]orthophosphate into the phosphatides and adenine nucleotides of mitochondria

The acetone precipitation procedure was applied to the problem of determining the initial rate of [^{32}P]orthophosphate incorporation into the fast-moving fraction of mitochondrial phosphatide. In these experiments the reaction mixtures were first prepared containing the mitochondria and all constituents except orthophosphate. The mixtures were attached to the manometers and equilibrated for 5 min at 10°. The phosphate buffer containing the [^{32}P]phosphorus was then added (this time was called zero time) and the reaction was followed manometrically by measuring the oxygen consumption. At the end of various time intervals the flasks were removed and an aliquot of 3.1 ml was removed and rapidly pipetted into 3.0 ml of acetone at a temperature of 45°. Simultaneous with the addition of the aliquot to the acetone, 0.5 ml of cold 20 % TCA was added to the remainder of the reaction mixture. Both solutions were immediately transferred to ice and kept cold. The lipid extracts were prepared from the acetone precipitates in the manner described. The TCA supernatant

TABLE II

THE INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO ADENINE NUCLEOTIDES AND PHOSPHATIDES BY RAT-LIVER MITOCHONDRIA

The reaction mixture contained the following: $2 \cdot 10^{-3}$ *M* DL-β-hydroxybutyrate, $5.0 \cdot 10^{-3}$ *M* [^{32}P]orthophosphate (pH 7.4), $3.3 \cdot 10^{-3}$ *M* ADP, $1.7 \cdot 10^{-3}$ *M* ATP, $6.7 \cdot 10^{-3}$ Tris buffer (pH 7.4). $6.7 \cdot 10^{-3}$ *M* $MgCl_2$, $5 \cdot 10^{-3}$ *M* KCl, $1 \cdot 10^{-2}$ *M* KF, and 1.2 ml of mitochondria. [^{32}P]orthophosphate contained approximately 500 μC/ml of [^{32}P]phosphorus Total volume was 4.65 ml. Temperature was 10°.

Flask	*Time (min)*	*Specific activity counts/min/μg P*				
		Inorganic P	*AMP*	*ADP*	*ATP*	*Phosphatide spots no. 8 and 9*
1	0	$43.4 \cdot 10^3$	$0.2 \cdot 10^2$	$0.02 \cdot 10^3$	$0.25 \cdot 10^3$	0
2	5	$43.6 \cdot 10^3$	$0.3 \cdot 10^2$	$1.70 \cdot 10^3$	$4.11 \cdot 10^3$	0
3	10	$40.7 \cdot 10^3$	$0.8 \cdot 10^2$	$3.59 \cdot 10^3$	$7.34 \cdot 10^3$	0.88
4	20	$39.4 \cdot 10^3$	$1.8 \cdot 10^2$	$6.34 \cdot 10^3$	$10.73 \cdot 10^3$	2.28
5	30	$37.7 \cdot 10^3$	$1.9 \cdot 10^2$	$7.09 \cdot 10^3$	$12.25 \cdot 10^3$	2.94
6	50	$30.8 \cdot 10^3$	$1.9 \cdot 10^2$	$7.84 \cdot 10^3$	$14.39 \cdot 10^3$	9.13

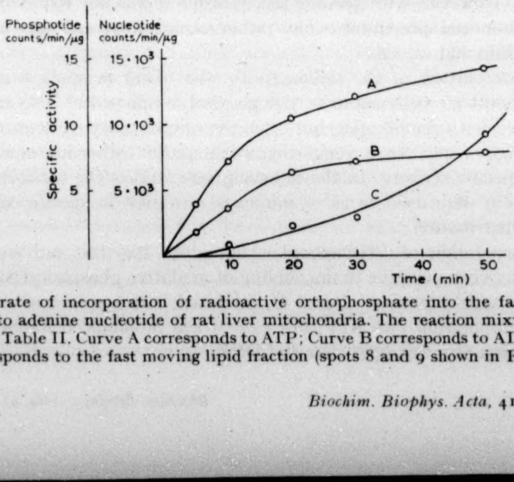

Fig. 2. Initial rate of incorporation of radioactive orthophosphate into the fast moving lipid fraction and into adenine nucleotide of rat liver mitochondria. The reaction mixture is the same as that given in Table II. Curve A corresponds to ATP; Curve B corresponds to ADP; and Curve C corresponds to the fast moving lipid fraction (spots 8 and 9 shown in Fig. 1).

fluids were prepared for chromatography of the adenine nucleotides and orthophosphate by washing with ethyl ether in order to remove the TCA.

The results of a typical experiment are given in Table II and Fig. 2. The specific activity of the inorganic phosphate given in Table II showed a slow decline during the course of the experiment. This was assumed to be due to the exchange of labeled orthophosphate in the medium with unlabeled pools of phosphate in the mitochondria. The ATP showed an initial rise in specific activity for the first 10 or 20 min and then leveled off to a considerably slower rate of increase. At the end of 50 min, the total ATP phosphorus had attained a specific activity which was only slightly less than half that of the inorganic phosphate. The ADP behaved in a very similar manner, but the rate of orthophosphate incorporation was little more than half the rate into ATP. As might be expected, the AMP showed a small incorporation of radioactivity.

In contrast to the adenine nucleotides, the combined phosphatide spots No. 8 and No. 9 showed no appreciable incorporation of radioactive phosphorus during the first 5 or 10 min. After 10 min radioactivity appeared in this fraction and rapidly increased with longer time intervals. Within 50 minutes there was no sign of leveling off.

DISCUSSION

A discussion of the rate of incorporation of [^{32}P]orthophosphate in the fast-moving phosphatide fraction of mitochondria must consider the incorporation into the nucleotides as well, particularly ATP, since this is the reference compound to which the incorporation into the lipids is compared if the lipid is an intermediate in oxidative phosphorylation.

The incorporation of [^{32}P]orthophosphate into ATP followed an apparently linear course during the first 10 min. After this time the rate of incorporation diminished markedly due to the decline of orthophosphate and phosphate-acceptor concentrations. ADP labeling followed a very similar pattern at a somewhat slower rate. The incorporation of [^{32}P]phosphorus into ADP is believed to be due to adenylate kinase activity. Adenylate kinase has been found in mitochondria by BARKULIS AND LEHNINGER[21] and KIELLEY AND KIELLEY[22]. At zero time the concentrations of AMP, ADP, and ATP were approximately equal. Since only ADP and a small amount of ATP were added, a transfer of phosphate groups between the nucleotides is believed to have occurred during the 5-min equilibration period before the addition of radioactive orthophosphate. This further indicates the presence of an active adenylate kinase. Throughout the first part of the experiment the ADP levels remained approximately constant. The increase in the ATP concentration appeared to be accompanied by a concomitant decrease in AMP concentration. These results may be explained by the simultaneous occurrence of ATP synthesis through oxidative phosphorylation and ADP resynthesis by the adenylate kinase reaction.

In contrast to the rapid rise in nucleotide activity in the first 10 min., the lipids showed a lag period during which no incorporation of radioactivity was measurable. This lipid fraction was found to contain several components and it was observed that the radioactivity may be in a minor component of high activity. The major portion of this fraction consists of polyglycerolphosphatide[3]. However, an intermediate in the phosphate esterification reactions of oxidative phosphorylation would, in all likelihood, rapidly obtain a specific activity which approached that of the orthophosphate and might be expected to occur in approximately the same concentrations as the cytochromes. CHANCE[23] has calculated $10^{-6}\,M$ as the approximate concentration of the cytochromes in a three fold diluted heart muscle preparation. It is probably approximately the same or somewhat higher in the isolated mitochondria preparations which were used in these experiments. On this basis a compound at the concentration of $10^{-6}\,M$ in the mitochondria preparation with the specific activity of the orthophosphate should produce, even if present as a minor component, a minimal specific activity in the total fast-moving phosphatide of about 5 counts/min/µg phosphorus. This specific activity was not reached in the lipid fraction until after 30 min. During this time, however, ATP was being rapidly labeled.

In addition, criteria for the establishment of a precursor-product relation between two components have been proposed by ZILVERSMIT *et al.*[24] for the case where a steady-state condition exists. During the time that the specific activity of both compounds is increasing, the specific activity of the precursor will always be greater than that of the product. Furthermore, the specific activity of the product will continue to increase until it equals the decreasing specific activity of the precursor. It would appear that neither criteria is met in the case of the fast-moving fraction and the ATP. It is considered therefore highly unlikely that an intermediate in oxidative phosphorylation could be present in the fast-moving phosphatide fraction even as a minor component. Unless the major part of the active phosphatide was lost in the acetone precipitation procedure, it would appear that phosphatides cannot function as intermediates in phosphate esterification prior to transfer to the nucleotides.

Earlier results on the rate of [^{32}P]orthophosphate incorporation into the phosphatides[25] obtained by centrifugal isolation of the mitochondria, followed by washing with KCl and direct extraction of the mitochondria pellet, showed a high incorporation after short incubation periods. In light of the experiments reported here it is assumed that the incorporation of orthophosphate into the phosphatide continued during the centrifugation and washing procedures in these experiments, after the supernatant fluid had been removed.

Although the active lipid fraction of mitochondria does not appear to participate directly in oxidative phosphorylation, its high turnover relative to the other phosphatides must be considered to have special significance. It is possible that this fraction may be an intermediate in the biosynthesis of the glycerolphosphatides[3].

ACKNOWLEDGEMENTS

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INTESTINAL TRANSPORT OF AMINO ACIDS STUDIED *IN VITRO* WITH L-[^{131}I]MONOIODOTYROSINE

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SUMMARY

The transport of L-[^{131}I]monoiodotyrosine by everted sacs of small intestine is dependent on active accumulation of this amino acid in the gut wall, the terminal ileum being the most active segment. Its transport is stimulated by glucose, which depresses the efflux of monoiodotyrosine from the mucosal surface of the intestine. Certain amino acids and potassium ion competitively inhibit monoiodotyrosine transport. α-amino acids of L-configuration with a free amino group and a long non-polar side-chain are the most effective inhibitors.

INTRODUCTION

Intestinal absorption of most amino acids occurs by way of an active transport mechanism[1-3]. Using the gut sac technique, Wiseman[3] has shown that certain amino acids are transferred from the mucosal to the serosal side of the small intestine against

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a concentration gradient. Little is known of the chemical mechanism involved in the active transport of amino acids, either by the intestine or by other tissues. However, certain structural requirements for transport of these compounds or for specific inhibition of their transport have been determined[3-7]. In the case of the intestine, these studies indicate that most of the neutral amino acids are well transported, whereas dicarboxylic amino acids and diamino acids are transported poorly or not at all; that L-amino acids are more readily transported than the corresponding D-isomers; and that transport of a given amino acid may be inhibited by certain other amino acids.

In the present study of amino acid transport by the small intestine, L-[^{131}I]-monoiodotyrosine (MIT) was used as the test compound. This amino acid is not metabolized appreciably by intestinal tissue. It is actively accumulated in the wall of the small intestine and transferred against a concentration gradient from the mucosal to the serosal side of everted gut sacs. Its transport is inhibited by certain amino acids, and the structural requirements for such inhibition have been determined. In addition, kinetic data were obtained which allowed a distinction between competitive and non-competitive inhibition.

EXPERIMENTAL

Methods

Wistar strain male rats weighing 150–250 g and fasted for 20 to 48 h were killed by decapitation, and the intestine quickly removed, everted, and tied into sacs approx. 2 cm in length according to the method of WILSON AND WISEMAN[8]. Except as noted in individual experiments, each sac was filled with 0.3 ml of medium containing 0.134 M NaCl, 0.011 M KCl, 0.008 M sodium phosphate buffer of pH 7.4, 0.001 M $CaCl_2$, 0.02 M glucose and 10^{-7} M [^{131}I]MIT; the sac was then immersed in 4.0 ml of the same medium in a 20-ml beaker. The beaker was covered with parafilm and incubated for 90 min at 37° in a Dubnoff shaker oscillating 120 times/min. At the end of incubation, the quantity of isotope in aliquots of the outer and inner fluids and in the tissue itself was determined in a scintillation counter. Sample counts generally exceeded 10 times the background. The volume of fluid recovered from inside the sacs was always slightly less than the initial 0.30 ml (generally from 0.24 to 0.28 ml), but it was assumed in calculating net transport that there was no actual change in volume. The product of the initial volume (0.30 ml) and the change in activity (counts/min/ml) of the inner fluid was taken as a measure of net transport of MIT. The results were then expressed as micromoles of MIT transported/100 mg wet wt. of tissue/90 min. In any single experiment the weights of gut sacs to be compared varied by less than 25 %.

Because of the variations in MIT transport in different parts of the small intestine, the mid-portion of the small intestine was used in experiments in which inhibitors or substrates were tested; every second or third consecutive sac served as a control. Transport was calculated as noted above and the result expressed as a percentage of the average transport of the two control sacs on either side. Each inhibitor or substrate was tested in this way in at least two different animals. In a few instances experiments were discarded because of appreciable differences between the two control values.

In experiments in which the uptake of MIT by open segments of intestine was measured, consecutive segments approx. 1 cm in length were taken from the mid-portion of the small intestine and incubated in the same manner as the intestinal sacs, except that the time of incubation was either 30 or 45 min.

The percentage of water in the intestinal tissue at the end of incubation averaged 84 % (83 to 85) of the wet wt. as determined by drying the tissue in a vacuum oven over P_2O_5 at 90° for 18 h.

Chromatography of the inner and outer fluids and of an extract of tissue was performed on Whatman No. 1 filter paper using butanol-acetic acid-water (78:5:17). The inner and outer fluids were used directly; the tissue was homogenized in the cold in a small volume of saline and protein precipitated with an equal volume of 10 % trichloracetic acid. The trichloracetic acid was extracted from the supernate with chloroform and an aliquot of the aqueous phase chromatographed. No activity was found in the chloroform extract.

Materials

[^{131}I]monoiodotyrosine was obtained monthly from Abbott Laboratories, Oak Ridge, Tennessee. Stable L-monoiodotyrosine was purchased from Mann Research Corp., New York, N.Y. and from Nutritional Biochemicals Corp., Cleveland, Ohio. DL-trifluoro-α-aminobutyric acid was kindly supplied by Dr. H. M. WALBORSKY, Tallahassee, Florida, and DL-2-aminoisobutanesulfonic acid was synthesized by the method of MCILWAIN[9]. All other compounds used as inhibitors or substrates were obtained commercially.

RESULTS

Recovery of MIT

At the end of the incubation period, over 90 % of the radioactivity in the inner fluid, the outer fluid, and in the tissue extract was found to migrate on chromatography with an R_F equal to that of MIT (Fig. 1). In the case of the tissue extract,

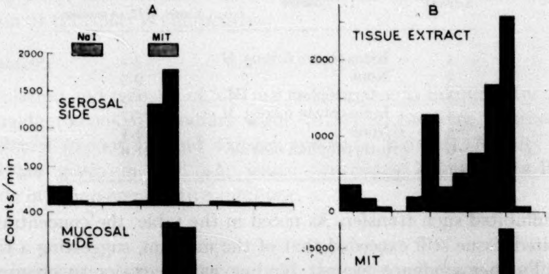

Fig. 1. Analysis of paper chromatograms of fluid from (A) the serosal and mucosal sides of everted gut sacs and (B) of tissue extract after incubation with ^{131}I MIT. Results with a sample of MIT treated in the same way as the tissue extract are also presented (B).

two peaks of radioactivity appeared on the chromatogram (in addition to a small amount of radioactivity at the origin), but a sample of MIT treated in the same way as the tissue gave identical peaks. It was concluded that the second peak was due to a change in MIT which occurred during the preparation of the tissue extract. The trichloracetic acid-precipitable protein of the tissue had no activity when the initial concentration of MIT in the incubation medium was $2 \cdot 10^{-7}\ M$, and less than 6 % of the total tissue activity when the initial concentration of MIT was $10^{-3}\ M$.

Tissue uptake and transfer of MIT

Incubation of everted gut sacs in standard medium with equal concentrations of MIT on the mucosal and serosal sides resulted in accumulation of this amino acid in the intestinal tissue and net transport into the fluid on the serosal side (Fig. 2). An initial rapid uptake of MIT by the tissue was followed by a rise in the concentration of MIT inside the sac. The time at which the rate of increase in MIT concentration on the serosal side was greatest (30–35 min) corresponded with the time of maximal tissue concentration.

That the tissue uptake of MIT is primarily an active process, rather than being due to binding, is indicated by the marked depression of MIT uptake in the presence of compounds which inhibit tissue metabolism or specifically inhibit MIT transfer across the intestinal wall (see below). The results with iodoacetate and L-tryptophan are presented in Table I. When studied with gut sacs, 0.0003 *M* iodoacetate partially inhibited transfer of MIT across the intestinal wall, and 0.01 *M* L-tryptophan completely inhibited such transfer. As noted in the table, the concentration of MIT in the inhibited tissue still exceeded that of the medium, suggesting a minor degree of binding. Further evidence against binding as the major mechanism in tissue accumulation of MIT are the findings, presented in Fig. 2, that the tissue concentration of MIT fell after 30 min while the concentration in the fluid inside was still rising. If the high concentration of MIT in the tissue were due to binding, one would expect the tissue concentration to have risen with the increase in concentration in the medium inside the sac.

TABLE I

EFFECT OF INHIBITORS ON TISSUE UPTAKE OF MIT

MIT uptake by open segments of small intestine. The segments were taken consecutively from the intestine of a single animal and incubated for 45 min at 37° in 4.0 ml of medium containing $10^{-7}\ M$ MIT.

Segment	*Inhibitor*	*Concn. MIT in tissue* / *Concn. MIT in medium*
1	Iodoacetate 0.0003 *M*	3.2
2	None	9.5
3	L-tryptophan 0.01 *M*	1.5
4	Iodoacetate 0.0003 *M*	2.7
5	None	8.2
6	L-tryptophan 0.01 *M*	1.6

The effect of increasing concentrations of MIT on its net transport is shown in Fig. 3 with the data plotted in the LINEWEAVER-BURKE form. In this instance net transport was determined after 90 min and therefore represents the average rate of transport over this time interval. In analogy with the MICHAELIS-MENTEN analysis of enzyme reactions, one can derive an apparent "K_m" and "v_{max}" from the LINEWEAVER-BURKE plot. Values for "K_m" and "v_{max}" differ from animal to animal, using comparable segments of gut. In four animals the apparent "K_m" values for the mid-portion of the small intestine were $4.6 \cdot 10^{-4}$ M, $2.2 \cdot 10^{-4}$ M, $2.0 \cdot 10^{-4}$ M and $7.9 \cdot 10^{-4}$ M; corresponding "V_{max}" values were 0.20, 0.21, 0.13 and 0.28 µmoles/100 mg/90 min. Since the rate of transport is not linear over the 90-min period and there is a continuous fall in outside concentration of MIT, these results obviously do not represent true MICHAELIS constants nor true maximal velocities. Nonetheless, as illustrated later, this treatment of the data is useful in determining whether or not inhibition of transport is competitive.

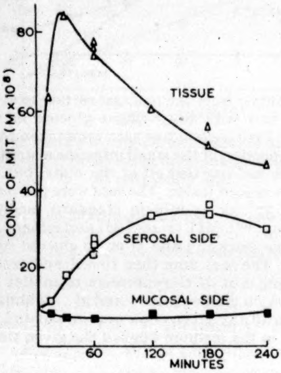

Fig. 2.

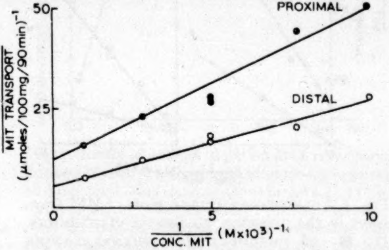

Fig. 3.

Fig. 2. Time curve of MIT uptake by everted gut sacs and of transfer to the serosal side. All sacs were taken from the mid-small intestine of the same animal.

Fig. 3. LINEWEAVER-BURKE plots of MIT transport by the proximal and distal small intestine of the same animal.

General inhibitors

Accumulation and transfer of MIT by the intestine is inhibited by anaerobiosis, sodium cyanide (0.0005 M), sodium azide (0.003 M), sodium fluoride (0.002 M), 2,4-dinitrophenol (0.0005 M) and sodium malonate (0.02 M). Results with 2,4-dinitrophenol are given in Fig. 4A, which illustrates LINEWEAVER-BURKE plots characteristic of a non-competitive inhibitor.

Substrates

Of the substrates tested, glucose was the most effective in enhancing MIT transport. In concentrations of 0.002 M, 0.01 M and 0.02 M it stimulated MIT transport to a varying degree in different animals (from 120 to 330% of control values). Fructose (0.01 M) was generally less effective (115 to 170%) and mannose (0.01 M) failed to stimulate transport in two of three animals (83, 100 and 150%). Lactate (0.01 M), pyruvate (0.0001 M), α-ketoglutarate (0.01 M), phosphoenolpyruvate (0.002 M), fructose diphosphate (0.002 M), phosphoglycerate (0.002 M), glycerol (0.01 M), acetate (0.01 M), propionate (0.01 M), butyrate (0.01 M), and

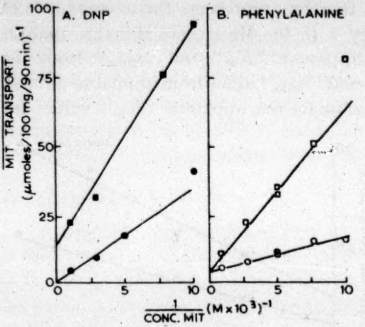

Fig. 4. Lineweaver-Burke plots of MIT transport in the presence and absence of inhibitors. ○, ●, no inhibitor; □, L-phenylalanine 0.0055 M; ■, 2,4-dinitrophenol 0.0003 M.

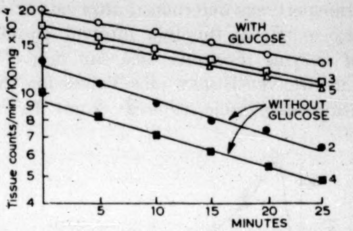

Fig. 5. Efflux from the mucosal surface of everted gut sacs with and without glucose in the medium. Five consecutive sacs were taken from the mid-portion of the small intestine of a single rat. Each sac was tied off at the ends, but no fluid was placed inside. The sacs were pre-incubated at 37° for 35 min in standard medium containing [^{131}I]MIT ($2 \cdot 10^{-6}$ M) and either 0.02 M glucose (sacs 1, 3 and 5) or no glucose (sacs 2 and 4). The sacs were then rinsed and transferred to 4.0 ml of fresh standard medium at 37° containing 0.01 M L-tryptophan to inhibit re-uptake of MIT, and either 0.02 M glucose (sacs 1, 3 and 5) or no glucose (sacs 2 and 4). An aliquot of the medium was counted every 5 min. Tissue counts/min at any given time were calculated by adding to the final tissue activity the total activity present in the medium beyond the given time.

valerate (0.01 M) had no effect. Succinate and citrate, each in a concentration of 0.01 M, inhibited MIT transport to less than 50 % of control values.

In order to characterize further the effect of glucose on the net transport of MIT, the efflux of MIT from the mucosal surface of the gut wall was determined in the presence and in the absence of added glucose (Fig. 5). Efflux of MIT is less when glucose is added to the medium; $t_{1/2}$ values derived from the plots of Fig. 5 are 40, 38 and 42 min in the presence of glucose, and 24 and 24 min in the absence of glucose.

Electrolytes

Potassium: Omitting K$^+$ from the medium did not affect MIT transport. However at KCl concentrations of 0.02 M and above (with equivalent reduction of NaCl concentration, which in itself had no effect), transport of MIT was depressed without a lowering of oxygen uptake. Since experiments with gut sacs gave erratic results for the degree of inhibition by high concentrations of K$^+$, open segments of intestine were used to obtain data for a Lineweaver-Burke plot. As shown in Fig. 6, the plots indicate that K$^+$ acts as a competitive inhibitor of MIT transport.

Sodium: MIT transport was completely abolished when NaCl was replaced with LiCl, KCl, $CaCl_2$, $MgCl_2$ or sucrose.

Calcium: The omission of Ca^{++} from the medium caused little or no reduction in MIT transport. However, in the presence of sodium Versenate (0.0003 M) transport was completely inhibited without a reduction in oxygen uptake. Increasing the $CaCl_2$ concentration to 0.015 M had no effect.

pH: During the standard 90-min incubation of gut sacs in 4.0 ml of medium containing 0.008 M phosphate buffer, the pH of the outside medium regularly fell from 7.4 to about 6.2. For this reason, in determining the effect of pH on MIT

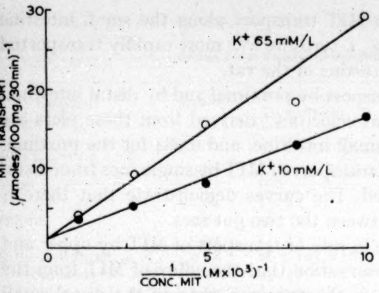

Fig. 6. Lineweaver-Burke plots of MIT transport in the presence of different concentrations of potassium ion.

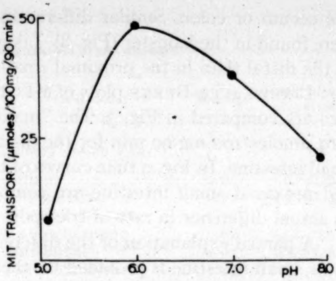

Fig. 7. Effect of change in pH on MIT transport. Consecutive everted gut sacs containing 0.30 ml of inner fluid were incubated for 90 min at 37° in 23 ml of medium contained in 125-ml Erlenmeyer flasks. The phosphate buffer concentration was 0.016 *M*. During incubation the respective pH changes were as follows: 7.9 to 7.6; 7.0 to 6.9; 6.0 and 5.1 unchanged. The first and last of the five sacs were run at pH 7.0 and gave identical results.

transport, larger volumes of a more concentrated buffer were used. The results are presented in Fig. 7. Maximal transport of MIT occurred at a pH of about 6.0.

Phosphate: When phosphate was omitted and the medium buffered with 0.008 *M* tris(hydroxymethyl)aminomethane of pH 7.4, MIT transport was unaffected.

Transport in different parts of the intestinal tract

MIT transport differed quantitatively in various parts of the small intestine (Fig. 8). It was consistently highest in the terminal ileum and lowest in the proximal jejunum. In the mid-portion of the gut, transport was fairly constant or gradually declined from the more distal to the more proximal region. No transport occurred in

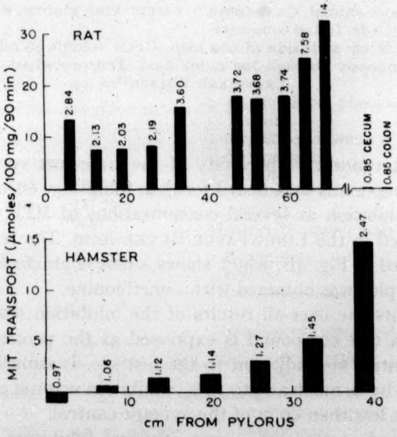

Fig. 8. MIT transport along the small intestine. Figures above the vertical columns are the ratios of the concentration of MIT on the serosal side to that on the mucosal side at the end of incubation.

the cecum or colon. Similar differences in MIT transport along the small intestine were found in the hamster (Fig. 8). Likewise, L-tyrosine was more rapidly transported in the distal than in the proximal small intestine of the rat.

LINEWEAVER-BURKE plots of MIT transport by proximal and by distal intestinal sacs are compared in Fig. 3. The "maximal velocities" derived from these plots are 0.19 μmoles/100 mg/90 min for the distal small intestine, and 0.084 for the proximal small intestine. In Fig. 9 time curves of the transport of MIT by single sacs from distal and proximal small intestine are compared. The curves demonstrate that there is an actual difference in rate of transport between the two gut sacs.

A partial explanation of the difference in rate of transport of MIT by upper and lower small intestine is provided by the observation that the efflux of MIT from the mucosal surface of the gut wall is greater in the proximal than in the distal small intestine (Fig. 10). The half-time values derived from the graphs are 28 and 33 min for the proximal segments and 40 and 43 min for the distal segments.

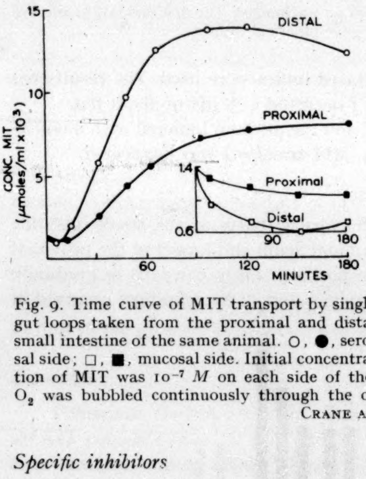

Fig. 9. Time curve of MIT transport by single gut loops taken from the proximal and distal small intestine of the same animal. ○, ●, serosal side; □, ■, mucosal side. Initial concentration of MIT was 10^{-7} *M* on each side of the loop. Outer volume 40 ml; inner volume 0.20 ml. O_2 was bubbled continuously through the outer fluid. The procedure was that described by CRANE AND WILSON[10].

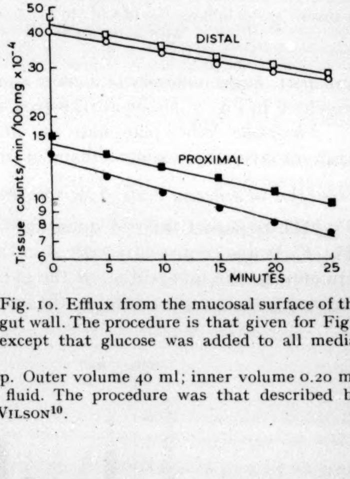

Fig. 10. Efflux from the mucosal surface of the gut wall. The procedure is that given for Fig. 5 except that glucose was added to all media.

Specific inhibitors

In order to determine the specificity of the transport system for MIT, amino acids and related compounds were tested for their inhibitory effects on MIT transport. In two instances inhibition at several concentrations of MIT was determined and the data were plotted in the LINEWEAVER-BURKE form. The results with L-phenylalanine are illustrated in Fig. 4B, which shows a plot characteristic of a competitive inhibitor. A similar plot was obtained with L-methionine.

Table II presents the over-all results of the inhibition studies. MIT transport in the presence of a test compound is expressed as the percentage of the average transport of two control sacs adjacent to the test sac. Inasmuch as the two control values often differed by as much as 5 to 10 %, inhibition was not considered significant unless transport was less than 90 % of the average control.

Of the compounds tested, the most effective inhibitors were L-tryptophan, L-methionine, L-ethionine, L-leucine and L-isoleucine. None of the four D-amino

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON MIT TRANSPORT

Compound	Molarity	MIT transport (% of control)
Aliphatic amino acids		
Glycine	0.01	106
	0.04	55, 65
L-alanine	0.01	46, 55
	0.04	11, 13
α-aminoisobutyric acid	0.01	103, 105, 116
DL-α-aminobutyric acid	0.01	29, 47
	0.02	0, 2, 9
DL-valine	0.01	53, 53
DL-norvaline	0.01	9, 11
	0.001	72
L-leucine	0.01	— 3, 6
L-isoleucine	0.01	3, 3
DL-isoleucine	0.01	6, 25
DL-norleucine	0.01	6, 7
	0.001	57
D vs. L amino acids		
L-alanine	0.01	46, 55
D-alanine	0.01	87, 89
L-phenylalanine	0.01	10, 27
D-phenylalanine	0.01	75, 104
L-valine	0.01	10, 12
D-valine	0.01	95, 106
L-methionine	0.01	— 3, — 1
D-methionine	0.01	86, 95
N-substituted amino acids		
Acetylglycine	0.04	84, 90
Sarcosine	0.04	97, 103
N-methyl-DL-alanine	0.08	87
Acetyl-DL-valine	0.02	109, 131
Chloracetyl-L-valine	0.01	93, 95
Acetyl-DL-methionine	0.02	77, 85
α-vs. β-and γ-amino acids		
L-alanine	0.03	12, 15
β-alanine	0.03	71, 79
DL-α-aminobutyric acid	0.02	0, 2
DL-β-aminobutyric acid	0.02	86, 102
γ-aminobutyric acid	0.01	85, 102
Sulfur-containing amino acids		
L-cysteine	0.01	19, 25
DL-cysteic acid	0.02	87, 100
DL-homocysteine	0.02	— 3, 1
L-methionine	0.01	— 5, — 1
L-ethionine	0.01	— 5, — 13
DL-methionine sulfoxide	0.02	42, 47, 53, 56
DL-methionine sulfone	0.02	49, 62, 71

Compound	Molarity	MIT transport (% of control)
Hydroxy amino acids		
L-serine	0.01	51, 66, 68, 69
DL-homoserine	0.02	29, 60, 66
DL-O-methylserine	0.02	14, 25
DL-phosphoserine	0.02	102, 103
L-threonine	0.01	60, 67, 84, 85
DL-allothreonine	0.02	23, 40
DL-phosphothreonine	0.02	118, 121
Basic amino acids		
DL-diaminopropionic acid	0.02	34, 53, 74
L-α, γ-diaminobutyric acid	0.01	92, 94
L-ornithine	0.01	93, 103, 115
L-lysine	0.01	73, 88
L-histidine	0.01	71, 82
L-arginine	0.01	60, 91
Dicarboxylic amino acids		
L-aspartic acid	0.01	102, 113
DL-asparagine	0.02	11, 35
L-glutamic acid	0.01	71, 80, 98, 124
DL-glutamine	0.02	21, 50
L-glutamic acid-γ-methyl ester	0.01	35, 62, 70
Aromatic amino acids		
L-phenylalanine	0.004	51, 57
L-tyrosine	0.004	47, 56
	0.0018	65, 81
DL-o-tyrosine	0.008	113, 120
DL-m-tyrosine	0.008	89, 94
	0.02	32, 43
L-3,4-dihydroxy-phenylalanine	0.004	109, 122
L-monoiodotyrosine	0.0018	22, 30
L-diiodotyrosine	0.002	95, 114
Tyramine	0.004	120
	0.01	89
Heterocyclic amino acids		
L-tryptophan	0.01	— 6, — 3
L-proline	0.01	71, 74
L-hydroxyproline	0.01	96
Miscellaneous compounds		
DL-2-aminoisobutanesulfonic acid	0.02	24, 27
Taurine	0.04	125
DL-γ-trifluoro-α-aminobutyric acid	0.02	18, 33
Phenylpyruvic acid	0.01	86, 100

acids tested showed more than slight inhibition of MIT transport in concentrations at which the corresponding L-isomers had a moderate to marked effect (Table II). N-substituted amino acids had essentially no inhibitory effect, though proline inhibited slightly. In contrast to the efficacy of the corresponding α-amino acids, β-alanine reduced MIT transport slightly, but β-aminobutyric acid and γ-aminobutyric acid did not reduce transport significantly. Although tyramine did not affect MIT transport, a carboxyl group did not appear to be necessary for inhibition since α-aminoisobutanesulfonic acid was effective. (This compound did not affect oxygen uptake by the gut sac.)

In the group of unsubstituted α-amino acids with aliphatic side chains, inhibition of MIT transport was greater the longer the side chain, up to norleucine. α-aminoisobutyric acid, which has an α-methyl group, did not decrease MIT transport whereas alanine was effective.

Among the sulphur-containing amino acids, a similar relationship between chain length and inhibition of MIT transport was found in a comparison of cysteine with homocysteine. (Each was undoubtedly oxidized in part to the disulfide.) Also of note is the marked difference in inhibition between cysteine and cysteic acid and between methionine and its sulfoxide and sulfone derivatives.

Of hydroxy α-amino acids L-serine and L-threonine were approximately equally effective as inhibitors. However, O-methylserine was more effective than serine, homoserine or threonine. In contrast, phosphoserine and phosphothreonine did not depress MIT transport.

Dicarboxylic and dibasic α-amino acids caused little or no inhibition of MIT transport, except for diaminopropionic acid. However, those derivatives of aspartic or glutamic acid in which the terminal carboxyl is substituted, as in the corresponding amides or glutamic acid-γ-methyl ester, depressed MIT transport.

Among the aromatic amino acids, the presence and position of substituents in the phenyl ring had considerable effect on the ability to inhibit MIT transport. Although tyrosine and phenylalanine had equal potency, *m*-tyrosine was a much less effective inhibitor and *o*-tyrosine, 3,4-dihydroxyphenylalanine and 3,5-diiodotyrosine did not inhibit in the concentrations used.

DISCUSSION

[^{131}I]monoiodotyrosine has certain advantages in the study of amino acid transport. Over 90% is recoverable unchanged after incubation with intestinal tissue, and less than 1% is incorporated into protein at concentrations of MIT suitable for transport studies. In addition, the radioactivity of tissue and media can be easily determined by direct counting, permitting the analysis of a large number of samples in a brief period.

The transfer of MIT against a concentration gradient from the mucosal to the serosal side of the everted gut sac appears to result from active accumulation of this compound by intestinal tissue and subsequent diffusion through the serosa. MIT is concentrated by the intestinal wall before net transfer to the serosal side occurs, and the time of maximal rate of transfer coincides with the time of highest tissue concentration of the amino acid. Thereafter the fall in tissue concentration is accompanied by a rise in concentration on the serosal side. In every instance there is a close parallel between net transfer of MIT in the gut sac and uptake of MIT by open segments of intestine; glucose, general inhibitors and the specific amino acid inhibitors all influence uptake and transfer of MIT to the same extent. Similarly, the differences in transfer rates along the gut parallel the differences in tissue uptake.

These results are consistent with the hypothesis suggested by AGAR, HIRD AND SIDHU[7] that the transfer of amino acids across the intestinal wall is dependent on an active transport mechanism located at the luminal surface of the mucosal cells. None of the above data can establish that the concentration gradient of amino acids is present at the mucosal surface of the epithelial cells rather than at another site in the intestinal wall. However, it has been reported that ingested tritiated leucine is concentrated to a greater extent in the mucosa than in the submucosa in small intestine of the mouse[11].

The reasons for the different rates of MIT transport in various segments of intestine are poorly understood. These differences might result from such factors as variations in the nature or amount of carrier, in available energy, or in the rate of back-diffusion of MIT. Present data do not permit any conclusions regarding the nature or quantity of a hypothetical carrier. Measurements of oxygen consumption, lactate production and glucose oxidation via the oxidative pathway indicate that all are lower in the distal than in the proximal small intestine of the rat or hamster[12, 13], whereas MIT transport is highest in the distal region. Therefore it seems unlikely that available energy is the determining factor. The differences in efflux of MIT from the upper and lower small intestine can account in part for the differences in net transport between these regions of the gut. One interpretation of the efflux data is that the permeability of the mucosa differs in various regions of the intestine, resulting in greater back-diffusion of MIT in the upper segments and therefore in a lower net transport.

The distribution of MIT transport along the small intestine of the rat and hamster is distinctly different from that reported for L-histidine[7], L-cysteine and L-cystine[14] in the rat. The explanation for this difference is not apparent, since inhibition studies suggest that these amino acids may compete for transport by a common mechanism.

The effect of potassium ion concentration on amino acid transport by ascites tumor cells has been studied by RIGGS, WALKER AND CHRISTENSEN[16]. These authors found that high concentrations of K^+ in the extracellular fluid or low concentrations of K^+ in the intracellular fluid led to diminished accumulation of amino acids by the cell. The mechanism of the inhibitory effect of K^+ is obscure. However, the finding in the present study that K^+ acts as a competitive inhibitor of MIT transport by the small intestine suggests that K^+ reacts reversibly with some part of the carrier mechanism for amino acid transport.

The stimulatory effect of glucose on MIT transport appears to be due, at least in part, to a diminution in efflux of MIT from the intestinal wall through the mucosal surface. Glucose (or presumably another suitable substrate) is apparently necessary to maintain an optimal barrier to the back-diffusion of the amino acid. Although the magnitude of the difference in efflux of MIT in the presence or absence of glucose seems adequate to account for the difference in net transport, the findings do not preclude the possibility that glucose also increases influx of MIT.

The transport of MIT was inhibited by certain amino acids and their derivatives, but not by others. At least two of the amino acids studied, L-phenylalanine and L-methionine, were shown to inhibit MIT transport competitively; whether the inhibition observed with other amino acids is competitive or non-competitive has not yet been determined. From the results of the inhibition studies the following generalizations may be made concerning the relationship between the structure of a compound and its ability to inhibit MIT transport: (a) the essential structure for effective inhibition is an α-amino acid of L configuration with an unsubstituted amino group and no ionized substituent in the side chain; (b) α-amino sulfonic acids are less effective inhibitors than corresponding α-amino carboxylic acids; (c) α-amino acids are much more effective than β-amino acids; the one γ-amino acid tested did not inhibit; (d) D-isomers have little or no effect; (e) amino acids with an ionized group in the side chain generally are not inhibitory and those with a non-ionized polar group in the side chain are poor inhibitors; (f) among amino acids with unsubstituted aliphatic side chains, inhibition is greater the longer the side chain from C_2 to C_6; (g) the length of the side chain is related to inhibitory effect; (h) α-methyl amino acids do not inhibit.

It is important to note that the inhibitory potency of an amino acid may not be directly related to its rate of transport. CHRISTENSEN *et al.*[4] in their studies of amino acid accumulation by Ehrlich ascites tumor cells, found in most instances a direct relationship between inhibition and transport, whereas WISEMAN[6] reported an inverse relationship in the rat small intestine. If the inhibitory potency of a compound is taken as a measure of its affinity for a carrier mechanism, the present data indicate that the carrier mechanism for neutral amino acids in the rat intestine has specificity for both ends of the molecule: a free amino group of L configuration close to an acidic group, and a non-polar group of the molecule rather remote from the charged amino acid end. This in turn suggests that alignment of the amino acid molecule across a lipid–water boundary may be a prerequisite to its reaction with a carrier.

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EFFECT OF MONOCHROMATIC ULTRAVIOLET LIGHT ON MACROMOLECULAR SYNTHESIS IN *ESCHERICHIA COLI*

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SUMMARY

Tracer techniques were used to follow the synthesis of protein and nucleic acids in *Escherichia coli*, strains *B* and 15T-, after the irradiation of exponentially growing cultures with monochromatic ultraviolet light (u.v.).

DNA synthesis is most sensitive to u.v., the observed effect being a delay in the initiation of ^{32}P incorporation into DNA. Doses above 150 ergs/mm² at 2652 Å result in cessation of DNA synthesis for a period greater than one normal division time.

The incorporation of ^{35}S into protein and of ^{32}P into RNA is linear with time after u.v. doses which stop DNA synthesis. Protein synthesis is inhibited more than RNA synthesis, and the dose-effect curves indicate a higher multiplicity for the effect of u.v. on RNA synthesis than on protein synthesis.

Action spectra for the effect of u.v. on protein synthesis and RNA synthesis are similar and follow a nucleic acid absorption spectrum.

The implications of these results, especially of linear synthesis, are discussed.

INTRODUCTION

The studies of many workers have shown that DNA synthesis is the cellular function most sensitive to the action of u.v. light[1-4]. However, there has been little quantitative evaluation of the effects on DNA synthesis as compared to the effects on synthesis of other macromolecular species in the bacterial cell. Such a comparison might be expected to provide clues to mechanisms of synthesis in the cell and should therefore be worthwhile.

SWENSON AND GIESE[5] have shown that much higher doses of u.v. are required to inhibit induced enzyme synthesis in yeast than are required to stop DNA synthesis. The action spectrum for the inhibition of enzyme induction implicated nucleic acid. HALVORSON AND JACKSON[6] studied the effect of u.v. on induced synthesis of β-galactosidase and on incorporation of ^{32}P into the nucleotides of RNA in yeast. The loss of capacity to utilize free amino acids in proteins paralleled the loss of the

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The following abbreviations will be used: Tris, tris(hydroxymethyl)aminomethane; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; TCA, trichloroacetic acid.

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ability to synthesize the enzyme. Doses of u.v. which inhibited RNA metabolism by 22 % were found to suppress enzyme formation by 95 %. DEERING AND SETLOW[7] in a quantitative study of the effect of u.v. on division in *E. coli B*, found no detectable effect on RNA and DNA synthesis after doses which inhibited division in most of the cells.

IVERSON AND GIESE[4] looked specifically at RNA and DNA synthesis in cultures of *E. coli B* after heavy incident u.v. irradiation. However, synthesis was followed for over a 4-h period, within which time the less affected cells may have had a chance to multiply several times (and repair mechanisms could operate), thus obscuring the initial effects on synthesis in the severely damaged cells. The incident doses used in their studies were much larger than those to be reported here because they made no correction for the absorption of light in the highly absorbing sample. It seemed to us that the most interesting time to take a detailed look at the effects of u.v. on synthesis in *E. coli* (with a division period of 50 min) should be in the early period of growth, immediately subsequent to the irradiation. COHEN AND BARNER[8], in their studies on unbalanced growth in bacteria, found that the permanent effects of inhibition of DNA synthesis were already manifest within one division period after this inhibition.

In the present studies exponentially growing bacteria were irradiated with monochromatic u.v. light. Special attention was paid to dosimetry, and macromolecular synthesis was examined closely in the critical period of "growth" immediately following the irradiation.

MATERIALS AND GENERAL PROCEDURES

Bacterial strains and growth conditions

The studies were carried out primarily on *Escherichia coli* 15_{T^-}[8], although several parallel experiments were performed on *Escherichia coli B* for comparisons as to generality.

The bacteria were cultured aerobically at 37° and at pH 7.4 in a synthetic medium containing in 1 l of water: 12 g Tris, 2.25 g KCl, 1.5 g NH_4Cl, 0.04 g $MgCl_2 \cdot 6H_2O$, 0.014 g Na_2HPO_4, 0.026 g Na_2SO_4, and 0.5 % glucose as carbon source. For balanced growth of strain 15_{T^-}, thymidine was added to the culture tube to a concentration of 0.8 μg/ml.

A 10-ml culture was inoculated from a nutrient agar slant and allowed to grow up overnight on a growth-limiting glucose concentration of 0.02 %. When glucose concentration was increased to 0.5 % the next morning, exponential growth resumed with little lag. Growth rate and cell concentration were estimated from the optical density of the culture at 625 mμ in a Coleman model 6A spectrophotometer. When the culture attained $8.8 \cdot 10^8$ cells/ml concentration, the bacteria were harvested by centrifugation for 2 min at 6700 × *g*. After resuspension in Tris buffer and a second centrifugation the cells were suspended in Tris to the desired concentration for the u.v. irradiations. Two centrifugations were found sufficient to remove essentially all thymidine from cultures of strain 15_{T^-}. Upon addition to the glucose–salt medium within a period of half an hour, the control cultures would resume exponential growth immediately.

Isotope incorporation and radioactivity assay

^{35}S incorporation into the "TCA-insoluble" fraction was used as a measure of protein synthesis[10]. Cells prepared as described above were pipetted into the isotopically labeled medium (containing 0.1 $\mu c/\mu g$ $^{35}SO_4$) at time zero. 0.2 ml aliquots were taken from the culture in duplicate at short intervals thereafter and pipetted into small test tubes. 5 ml ice-cold 5 % TCA was added to each tube and the contents were then emptied into collodion filters (Schleicher & Schuell, 27 mm diam., coarse porosity) to collect the "TCA-insoluble" fraction[9,10]. The filters on a suction filtration apparatus were then rinsed with 5 ml distilled water before they were allowed to dry in air. The radioactivity on the dried filters was determined by conventional GM-counter methods employing an Amperex 1.4 mg/cm^2 thin mica window counting tube, placed in a ½-inch thick lead shield, at a distance of 3 mm from the filter surface. Counting times were set so as to allow roughly 5% variation from statistical fluctuations in counting rate. Background counting rate and the radioactivity adsorbed to the filters from the medium, as determined prior to incorporation experiment, were subtracted from the observed rate to give the "TCA-insoluble" rate.

^{32}P incorporation was used to follow nucleic acid synthesis. An adaptation of the SCHMIDT-THANNHAUSER separation procedure[11] to the collodion filter technique of ROBERTS *et al.*[10] permitted the simultaneous observation of ^{32}P incorporation into the RNA and the DNA fractions of the same sample from the culture. The details of this procedure and an estimate of reliability are reported elsewhere[9,12]. Bacteria were grown in 10 ml Tris medium containing 0.04 $\mu c/\mu g$ $^{32}PO_4$ and, as for the ^{35}S assay, 0.2 ml aliquots were withdrawn in duplicate for the determination of incorporation into the RNA, DNA, and phospholipid fractions.

U.v. irradiations

A large water-prism monochromator utilizing a 250 W low-pressure mercury arc lamp was used for the irradiations[13]. The intensity was controlled, within limits, by the exit-slit width. The resolution of the instrument allowed an 8 mm separation between the 2805 Å and 2652 Å lines at the position of the exit-slit. Scattered radiation at wave-lengths above 2400 Å amounted to less than 1 % of the intensity. The intensity at the wave-length to be used was determined at the position of the sample tube prior to the irradiation, by means of a calibrated G1-955 phototube with a 1 cm^2 mask. The phototube output was measured by a d.c. amplifier and read on a calibrated meter scale. The phototube was calibrated over the u.v. spectral range against a thermopile whose output had been calibrated by standard lamp.

Irradiations were performed on bacteria from an exponentially growing culture, suspended in Tris buffer to a concentration of about $2.5 \cdot 10^8$ cells/ml. The percent transmission of the sample to be irradiated was determined prior to irradiation, in a Beckman model DU spectrophotometer, and the average effective intensity was then calculated by the method of MOROWITZ[14]. An additional correction factor of 0.9 accounted for the light scattered from the front window of the irradiation cell. 2.3 ml of the bacterial suspension was placed in a 1 cm path-length quartz cell, and the sample was stirred manually by means of a 2 mm twisted glass stirring rod during the irradiation. Irradiation times were of the order of 1 to 5 min. The irradiated samples and the unirradiated control sample were kept at 37° before, during, and after the irradiation. No effect on isotope incorporation was observed as due to keeping the *E. coli* cells in buffer at 37° for up to half an hour before or after the irradiation. Samples were kept in subdued light until at least 20 min of growth in the Tris medium after the irradiation, to minimize photoreactivation effects. All irradiations reported here were performed at 2652 Å unless otherwise specified.

CONTROL EXPERIMENTS

Preliminary incorporation studies

Before undertaking a study of macromolecular synthesis by the incorporation of radioisotopes into bacteria which have been treated in various ways, it is important to have a thorough knowledge of the kinetics of isotope incorporation by normal bacterial cells. This has been examined in detail by ROBERTS *et al.*[10] for both $^{32}PO_4$ and for $^{35}SO_4$ labeling. However, it was necessary to verify their kinetics for the conditions and media to be used in the present studies.

A simple model for the incorporation of ^{32}P (from $^{32}PO_4$) into the TCA-insoluble fraction of exponentially growing cells can be represented by the following expression[10]:

$$Z = [P_0 e^{at}/(b - a)]\,[b(1 - e^{-at}) - a(1 - e^{-bt})]$$

in which Z represents the total incorporated activity per unit volume of culture at time, t, after the addition of isotope. a is the growth constant, and b is the pool "equilibrium" constant, which is dependent on the rate of formation of small molecular weight precursors as well as on the rate of exchange between the pool and the external environment. P_0 is a constant dependent on the initial concentration of cells, and it represents the extrapolation of the logarithmic incorporation rate obtained at large values of t, to the ordinate at $t = 0$.

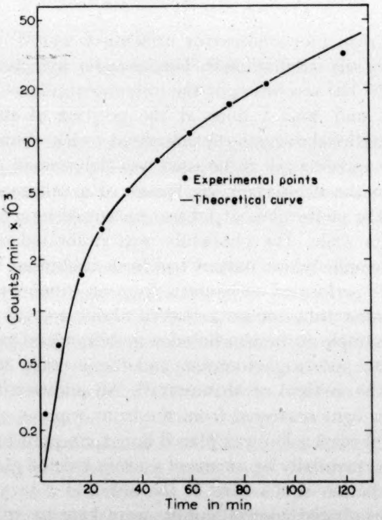

Fig. 1. The incorporation of ^{32}P into the TCA-insoluble fraction of normal exponentially growing *E. coli B* in Tris medium at 37°.

The model was verified experimentally as shown in Fig. 1, where data on the incorporation of ^{32}P into the TCA-insoluble fraction of exponentially growing *E. coli B* is presented. The growth constant was determined from a semilog plot of colony count *vs.* time. The constants P_0 and b were then evaluated by trial and error to achieve the best fit to the theoretical expression. The value obtained for b corresponded to a "half-life" of 4.5 min for the attainment of the pool steady-state condition. Effectively this means that a logarithmic rate of incorporation into the TCA-insoluble fraction is not approached until about 30 min from the time at which the isotope is added to the growth medium. A similar picture but with a much shorter "half-life", was obtained for the incorporation of ^{35}S (from $^{35}SO_4$) into the TCA-insoluble fraction of exponentially growing *E. coli B*. This effect must be borne in mind in studies of synthesis rates in the cell as measured by the incorporation of radioisotopes.

Turnover

E. coli 15_{T-} were grown in $^{32}PO_4$-containing medium until highly labeled. Cells were then separated from the labeled medium by centrifugation and were resuspended in Tris buffer. A dose of 400 ergs/mm² u.v. at 2652 Å was administered. Then equal aliquots of irradiated and control samples were added to non-labeled Tris medium. The radioactivity per unit volume of culture (TCA-insoluble fraction) was determined at intervals by the filter method. The procedure was repeated using $^{35}SO_4$ as label. ^{32}P loss amounted to less than 0.5 % per hour in the first hour for both control and irradiated cultures, although after an hour of growth activity was lost rapidly from the irradiated cells. Roughly 1 % loss of ^{35}S was obtained in the first hour by both control and irradiated cultures, and a similar drop in ^{35}S content in the irradiated cultures occurred after this period. It was concluded that turnover and autolysis effects are negligible in the experiments to be described, since incorporation studies were carried out within the first hour after irradiation.

Reciprocity

In studies on the effects of different wave-lengths it is necessary either to use the same intensity for irradiation at each wave length, or to demonstrate that reciprocity holds over the range of intensities to be used. Within experimental error the same incorporation curve (for either ^{32}P or ^{35}S) was obtained with *E. coli* 15_{T-} after a u.v. dose of 200 ergs/mm² at 2652 Å as intensity was varied over a factor of four. A further check on reciprocity was obtained at 2301 Å and at 2400 Å where the data obtained using the low intensity lamp were in agreement with those obtained using a General Electric H-6 high pressure mercury arc which gave a ten times higher intensity.

EXPERIMENTAL

Colony-forming ability in E. coli 15_{T-}

For purpose of comparison with the effects on molecular synthesis, data were obtained on the effect of u.v. on colony-forming ability in *E. coli* 15_{T-}. Immediately after irradiation at 2652 Å, dilution series (to give about 200 colonies per plate) were carried out in Tris buffer at room temperature and aliquots were spread on Difco (23 g/l) nutrient agar plates. Incubation at 35° was begun immediately after plating. The resulting dose–survival curve for colony-forming ability is shown in Fig. 2, and indicates 37 % survival after a dose of 65 ergs/mm². Using a different plating procedure (delay after irradiation and lower incubation temperature) SETLOW AND CUMMINGS (unpublished) obtained a 37 % survival dose of 50 ergs/mm². However, it is well known that post-irradiation treatment affects the viability of irradiated bacteria[15]. DEERING[16] obtained 37 % survival of colony-forming ability in *E. coli B* after doses of 30 ergs/mm² or 10 ergs/mm², depending on plating procedure used.

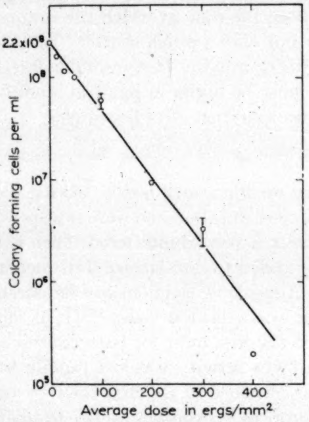

Fig. 2. A dose-survival curve for colony-forming ability of *E. coli* 15_{T-} after u.v. irradiation at 2652 Å.

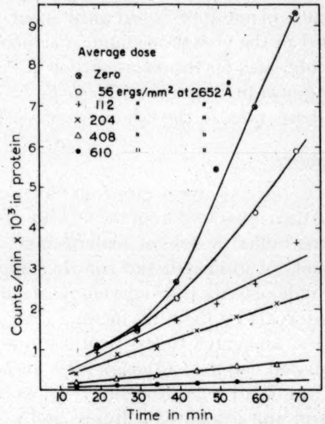

Fig. 3. Sample data on the incorporation of ^{35}S into the TCA-insoluble fraction of *E. coli* 15_{T-} after different doses of 2652 Å radiation.

Protein synthesis

Sample data from a ^{35}S-incorporation run with *E. coli* 15_{T-} after different doses of u.v. at 2652 Å are shown in Fig. 3. The control culture and the five different irradiated cultures were started in labeled medium simultaneously, after the irradiation. The filtering apparatus, holding six filters at once, allowed sampling from alternate groups of three cultures (in duplicate) at the same times, providing control on growth conditions for purposes of comparing effects of different doses. All points in the incorporation data represent a numerical average of two samples taken from the culture at the same time.

Note that incorporation into the TCA-insoluble fraction appears to be linear after doses above about 150 ergs/mm². The linear synthesis of protein after high doses of u.v. was verified in a more detailed check, using *E. coli B*. This strain does not lyse after high doses of u.v., so growth can be followed for a longer period after the irradiation. In Fig. 4 are shown the data for the incorporation of ^{35}S into the TCA-insoluble fraction of *E. coli B* after a dose of 150 ergs/mm². In this experiment, in contradistinction to the others described, the bacteria were grown for several generations in the radioactive medium before irradiation. Thus, the problem of the attainment of pool equilibrium could be disregarded, even at short times after the irradiation. The data in Fig. 4 are plotted both on a linear scale to show the linear incorporation after u.v. and on a logarithmic scale to show the exponential incorporation in the unirradiated control culture. KELNER[17] has interpreted data for total cell mass similar to those shown in Fig. 4b, to indicate that irradiated cultures show an abrupt change in exponential rate of mass increase. We would rather interpret our data as indicating linear rather than two exponential rates. It is important to note that the ^{35}S-incorporation rate for a dose of 150 ergs/mm^2 is the *same* as that in the control culture immediately after the irradiation. As time goes on, the incorporation rate in the control culture increases exponentially, but the rate in the irradiated culture remains the same. Larger doses than 150 ergs/mm^2 yield linear rates of incorporation which are less than the initial control value. Smaller doses give rise to incorporation curves which are between linear and exponential.

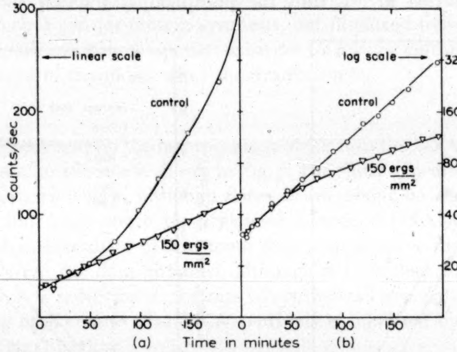

Fig. 4. Sample data on the incorporation of ^{35}S into the TCA-insoluble fraction of *E. coli B* after 150 ergs/mm^2 at 2652 Å. a. The data on a linear scale. b. The same data on a logarithmic scale.

For a check on the use of ^{35}S incorporation as a measure of protein synthesis in *E. coli* 15$_{T-}$ after irradiation, the effect of u.v. (two different doses) was determined on the incorporation of [^{35}S]methionine and of [^{14}C]proline. The results obtained were consistent with those obtained by using $^{35}SO_4$ as label after the same doses of u.v.

The linear incorporation curves of Fig. 3 allow the effects of different doses to be compared by comparing the slopes of these incorporation curves. Taking into account the pool saturation effect mentioned earlier, it seemed best to measure these slopes at a time 35 min after addition of cells to labeled medium. The slopes of the exponential control and the near-exponential curves obtained after low doses were approximated by the tangents to the curves at the 35-min point. It should be noted that any method of comparison at low doses is somewhat arbitrary since the shapes of the incorporation curves change with dose in this region. It is easier experimentally to determine the slopes at 35 min than at zero time, even if the cells were fully labeled to eliminate the problem of pool equilibrium.

A dose–effect curve for protein synthesis was constructed by plotting the relative incorporation rates at 35 min (compared to the unirradiated control) after different doses. A simple exponential function resulted from this analysis, yielding a 37%

survival dose of 160 ergs/mm² for *E. coli* 15_{T-} *E. coli B* also gave a first-order dose-survival curve for protein synthesis, but with a 37% survival dose of 210 ergs/mm². The shape of the dose–effect curve is arbitrary for low doses. If rates had been compared at zero time, no effect would have been observed until doses greater than 100 ergs/mm² for strain 15_{T-} and 150 ergs/mm² for strain *B* were given. However, the slope of the straight line portions would still be the same as shown in Fig. 5.

Within experimental error the same survival for protein synthesis in *E. coli* 15_{T-} was found in cultures growing in the presence and in the absence of thymidine subsequent to the irradiation.

Phospholipid synthesis

The incorporation of ^{32}P into the phospholipid fraction showed too much fluctuation to be used as a valid measure of synthesis in this fraction after u.v. treatment of *E. coli* 15_{T-}. Qualitatively the incorporation appeared to be exponential, even after doses of u.v. above 150 ergs/mm². A 37 % survival dose for phospholipid synthesis was estimated at between 400 and 600 ergs/mm², based on a comparison of rates of incorporation of ^{32}P into the phospholipid fraction, measured as tangents to incorporation curves at the 35-min point.

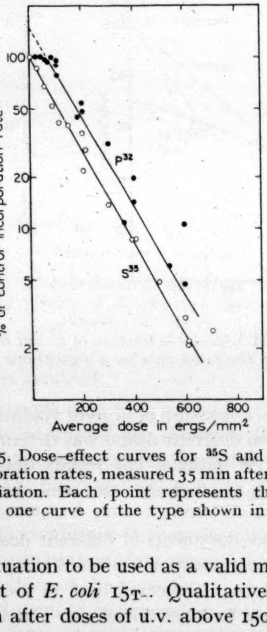

Fig. 5. Dose–effect curves for ^{35}S and ^{32}P incorporation rates, measured 35 min after 2652 Å irradiation. Each point represents the data from one curve of the type shown in Fig. 3.

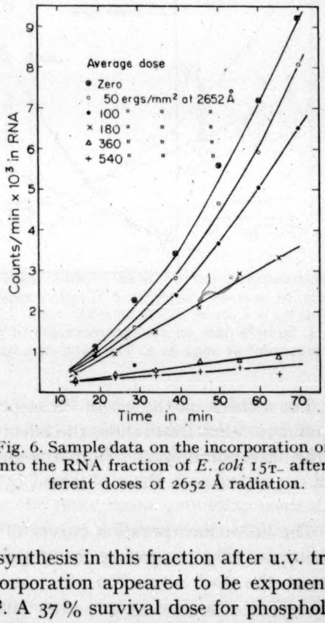

Fig. 6. Sample data on the incorporation of ^{32}P into the RNA fraction of *E. coli* 15_{T-} after different doses of 2652 Å radiation.

RNA synthesis

Sample data from an experiment on ^{32}P incorporation into the RNA fraction of *E. coli* 15_{T-} after different doses of u.v. are shown in Fig. 6. The incorporation is linear after doses above 150 ergs/mm². The slopes of the incorporation curves were measured, as with the ^{35}S studies, at the 35-min point. The resulting dose–effect curve (Fig. 5) for RNA synthesis does not give the simple exponential relationship found for protein synthesis, nor does it follow multiple target kinetics in the low dose range. (In a comparison of eight irradiations with doses below 60 ergs/mm^2, the rates of incorporation at 35 min by the irradiated samples were always within experimental error of that of the controls.) Even though the dose–effect curves are again somewhat arbitrary in shape for low doses, it is evident that RNA synthesis is considerably *less* sensitive to u.v. than is protein synthesis.

Dose–effect curves at different wave-lengths showed the same multiplicity found at 2652 Å, the exponential part of the curves extrapolating back to an intercept of roughly 1.5. Similiar kinetics were found for *E. coli B*. After irradiation at 2652 Å the 37 % survival dose was 240 ergs/mm^2 for *E. coli* 15$_{\text{T}-}$ and 280 ergs/mm^2 for *E. coli B*. The exponential parts of the dose–survival curves for RNA synthesis were parallel to those obtained for protein synthesis, but displaced from them.

RNA synthesis was found equally sensitive to u.v. in cultures growing in the presence or absence of thymidine after the irradiation[18].

DNA synthesis

A sample experiment on the incorporation of ^{32}P into the DNA fraction of *E. coli* 15$_{\text{T}-}$ cells after u.v. irradiation is shown in Fig. 7. The effect appears to be a temporary inhibition of DNA synthesis, although doses below about 20 ergs/mm^2 seemed to have no effect and doses above 150 ergs/mm^2 destroyed DNA-synthesizing ability permanently. It is difficult to obtain more than a qualitative idea of the effect on DNA synthesis from the data obtained, although it is evident that the effect does not involve merely a reduction in the rate of synthesis. These data are in agreement with the finding of KELNER[1] that DNA synthesis is inhibited by u.v. for a period roughly proportional to dose.

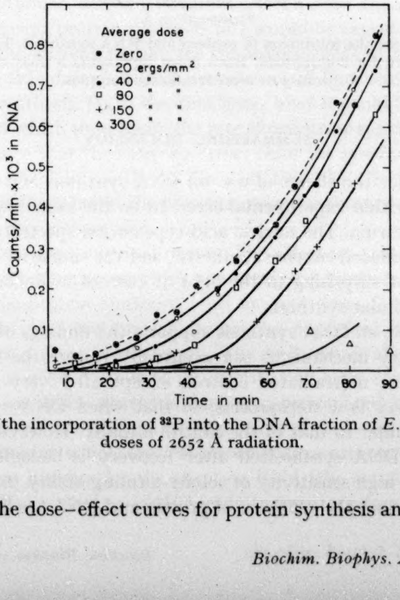

Fig. 7. Sample data on the incorporation of ^{32}P into the DNA fraction of *E. coli* 15$_{\text{T}-}$ after different doses of 2652 Å radiation.

Action spectra

The shapes of the dose–effect curves for protein synthesis and for RNA synthesis were shown to be independent of the wave-length, but the exponential slopes of these curves were wave-length dependent. The relative sensitivity to different wave-lengths may thus be expressed as the reciprocal of the 37 % dose obtained from the exponential portion of the dose–effect curves at these wave-lengths. The sensitivity at each wave-length can be expressed in terms of cm² per incident quantum. Fig. 8 shows the u.v. action spectra obtained in this fashion. An absorption spectrum for DNA is given for comparison.

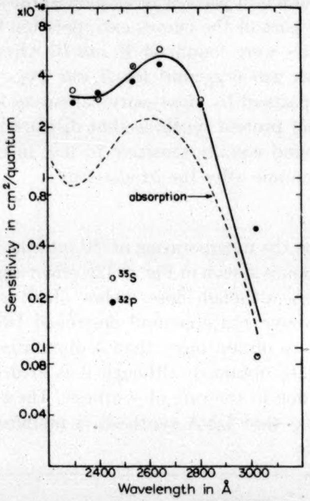

Fig. 8. Action spectra for the inhibition of protein and RNA synthesis. The absorption spectrum of nucleic acid is shown for comparison. Each point represents a dose–effect curve constructed from 3 or more irradiated samples.

SUMMARIZING DISCUSSION

The action spectra for the effects of u.v. on RNA synthesis and on protein synthesis have been shown, within experimental error, to be the same and to follow a nucleic acid absorption spectrum. The nucleic acid-type action spectrum has been found by others workers for induced enzyme synthesis[5], and the similarity of protein and RNA action spectra is not surprising in the light of current evidence for the association of nucleic acid in cellular synthesis.

The effect of u.v. on DNA synthesis supports the findings of other workers. It is evident that recovery mechanisms can eventually restore the DNA synthesis rate to nearly that of the unirradiated control, except after very high doses. Also, it appears that recovery is a step-process, in that when DNA synthesis resumes, it does so at a rate similar to that of the control initially. However, it is very possible that much of the DNA synthesized after recovery is biologically non-functional, as suggested by the high sensitivity of colony-forming ability to u.v.

It is significant that the synthesis of protein and RNA are linear after u.v. doses which inhibit DNA synthesis for appreciable periods of time. Linear synthesis of RNA has also been observed in *E. coli* 15_{T-} when DNA synthesis is inhibited by thymine deficiency[9], although protein synthesis continues for a period at an increasing rate under this condition[12]. R. B. ROBERTS (personal communication) has evidence from studies with this strain that *particulate* RNA synthesis is constant in the absence of DNA synthesis, but that both soluble RNA and protein synthesis rates increase. Such results could be explained by supposing that the rate of (particulate) RNA synthesis is determined in part by the number of intact DNA "units" in the cell. This idea is supported by the fact that when DNA synthesis resumes in a culture after a low dose of u.v., the RNA synthesis rate increases. One might ask if the linear rates observed after u.v. are not merely a fortuitous combination of exponential rates in some cells, counteracted by decreasing rates of synthesis in other, more seriously damaged, cells? However, then one might expect to see decreasing rather than linear rates of synthesis after still higher doses of u.v. Our results show that this is not the case, linear rates being found in all cases where DNA synthesis is stopped.

The output in synthesis of RNA and protein per unit DNA could be controlled partially by the supply of precursors. Thus, linear synthesis would be expected in cases in which precursors were supplied at a constant rate. A case of this type may have been observed by COHEN[19] who observed linear growth in *E. coli K*-12 in the presence of high valine concentrations. The rate of precursor synthesis could have been limited by the concentration of enzymes existing *before* the cells began to produce non-functional enzymes containing valine as an amino acid analogue.

A striking difference in the effect of inhibition of DNA synthesis by u.v. and by thymine deficiency is the fact that a *linear* rate of protein synthesis is observed in the former and an *increasing* rate is found in the latter, RNA synthesis rates being constant in both cases. Why does protein synthesis appear to be less sensitive than RNA synthesis to thymine deficiency, but more sensitive than RNA synthesis to u.v. action? An increasing protein synthesis rate would be expected from the increase in RNA, assuming the current picture for the involvement of ribosomes in protein synthesis. It may be that damaged DNA specifies much "nonsense" RNA which cannot sustain protein synthesis. Or at the next lower level it could be the protein which is non-functional and which then limits the rate of precursor synthesis. MCFALL *et al.*[20] have provided evidence that the *integrity* rather than the *synthesis* of bacterial DNA is a precondition for protein synthesis. The fact that the effect of u.v. on protein and RNA synthesis is the same in the presence as in the absence of thymine in *E. coli* 15_{T-} would support this picture also.

Now, consider the dose–survival curves. The effect on protein synthesis can be described by single-hit kinetics in which the surviving fraction is equal to $N/N_0 = e^{-aD}$, where N_0 is the protein synthesis level for zero dose, D is the u.v. dose, and a is the inactivation cross section, which may be expressed in terms of area per incident quantum at a given wave-length. The 37 % survival of protein synthesis after 160 ergs/mm² at 2652 Å leads to an inactivation cross section of 5 Å² by this analysis. The effect on RNA synthesis can be approximated by a multiple-target analysis, although as we have pointed out, the *shape* of the dose–effect curve depends somewhat on the time at which the ^{32}P incorporation rates are compared. But, if we assume two-target kinetics for the effect on RNA (the actual intercept was 1.5), the relationship $N/N_0 = 1-(1-e^{-aD})^2$ leads to two targets of cross section 5 Å², each of which must be inactivated. If the suggested conclusion that the two strands of a DNA molecule are involved, is true, then it would appear that damage to one strand is sufficient for unit effect on protein synthesis, but that both strands must be damaged for unit effect on RNA synthesis. The implications of such a model are perplexing at best.

Assuming that DNA is the structure involved in u.v. action and taking the quantum yield of 10^{-2} for damage to DNA[21], the absorption cross section for the sensitive unit at 2652 Å should be 500 Å^2/photon. (Quantum yield = inactivation cross section/absorption cross section.) Since the u.v. absorption cross section for nucleic acid is about 20 times that for protein and since there is about 6 times as much RNA as DNA in the exponentially growing *E. coli* $15_{\text{T-}}$ cell[12], the absorption at 2652 Å is due predominantly to the ribosomes which contain most of the RNA in the cell. The u.v. absorption (corrected for scattering) at 2652 Å by $2.5\cdot 10^8$ cells/ml gives an optical density of 0.45 (ref. 12). Assuming 50,000 ribosomes per cell leads to a concentration of $12\cdot 10^{12}$ ribosomes/ml ($2\cdot 10^{-8}$ moles/l). As an approximation say that these ribosomes accounted for 0.4 units of the observed optical density. Then the molecular extinction coefficient for the ribosomes would be $0.2\cdot 10^8$, which leads to an absorption cross section of about 10^{-13} cm^2/photon. The quantum yield for protein synthesis (assuming ribosomes to be the active centers for this synthesis) is then

$$\frac{5\cdot 10^{-16}\ \text{cm}^2/\text{photon (Inact. cross sect.)}}{10^{-13}\ \text{cm}^2/\text{photon (Absorption)}}$$

or $5\cdot 10^{-3}$, which is within a factor of 2 of the quantum yield for damage to DNA. It is suggested that the primary sites of action of u.v. are the ribosomes, but that the primary damage is to the DNA which may be associated with the ribosomes.

ACKNOWLEDGEMENTS

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FRACTIONATION OF RIBONUCLEIC ACIDS WITH PHENOL

I. TWO METABOLICALLY DISTINCT CLASSES OF RIBONUCLEIC ACIDS IN ANIMAL CELLS

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SUMMARY

In rabbit lymphatic cells and rat livers incorporating ^{32}P-orthophosphate *in vitro* or *in vivo*, the specific activity of RNA released into aqueous phase by treatment with phenol was considerably lower than that of total RNA and rather close to that of DNA. Existence of a minor RNA fraction with exceedingly high specific activity in the residue remaining in the phenol phase was demonstrated in the liver. Guanylic acid content was high in the phenol-released RNA fraction and low in the residual RNA fraction. In the regenerating liver the specific activity of phenol-released RNA seemed to be augmented and to decline earlier than that of DNA.

It has been assumed that animal cells contain two chemically and metabolically distinct classes of RNA which may be roughly fractionated by phenol treatment of the cellular material; the one (class I) that predominates in amount may, like DNA, be metabolically rather inert and duplicated only once during the mitotic cycle in the steady state, while the other (class II) is turned over at a considerably high rate.

INTRODUCTION

It is widely accepted that RNA of animal cells is highly heterogeneous. Support to such a view is given by the work of a multitude of investigators on the fractionation of RNA of whole cells[1], nuclei[2-5], cytoplasmic particulate elements[6-8] and supernatants[7-9] into components differing in metabolic activity. While studying the incorporation of ^{32}P-orthophosphate into nucleic acids of animal cells, we have also found that the extraction of RNA with aqueous phenol according to the method of KIRBY[10] resulted in a clear-cut fractionation of RNA of the whole cell. This finding was remarkable in two respects. First, KIRBY's phenol method is now extensively utilized for obtaining RNA from various sources. Secondly, one of the fractions obtained had a specific activity close to that of DNA, while the other fractions showed an exceedingly high specific activity. In this study the various nucleic acid fractions

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNase, deoxyribonuclease.

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labelled with ^{32}P-orthophosphate *in vitro* or *in vivo* were first hydrolysed and separated into constituent mononucleotides. The quantitatively recovered nucleotides were then freed of radioactive contamination for the determination of the specific activity. Such a procedure precluded a further fractionation of nucleic acids during the course of analysis.

Some preliminary accounts on this subject have already appeared[11],[12]. Results of a similar study on the Ehrlich ascites carcinoma have also been reported by one of the authors[13].

EXPERIMENTAL

Materials. Suspensions of lymphatic cells were prepared from rabbit appendices and Peyer's patches as described before[14]. Liver slices were prepared manually from normal or regenerating livers of the rat. Partial hepatectomy was conducted as in a previous study[15]. Sufficient material for one experiment was 10^{10} lymphatic cells or 15 g of liver slices.

In vivo *experiment.* Rabbits receiving 300 μC each of $Na_2H^{32}PO_4$ by an intravenous injection were killed 2 h later, and lymphatic cells were isolated as above.

In vitro *experiments.* Incubation of lymphatic cells was conducted in Ca-free Tyrode solution with 5 μC of $^{32}PO_4$/ml as previously described[14]. The incubation of liver slices was made as in previous experiments[15] in Ca-free Ringer-bicarbonate buffer containing 2–5 μC of $^{32}PO_4$/ml in a closed vessel under 95 % O_2 and 5 % CO_2 atmosphere. Incubation lasted 1–2 h.

Total RNA and DNA. The fresh or incubated lymphatic cell suspensions were processed without separating the cells from the medium or homogenizing. The liver slices were first centrifuged off and, after decanting the medium, homogenized in 3 volumes of distilled water in a Potter–Elvehjem homogeniser. One fourth to 1/5 of the material was used for the separation of total RNA and DNA, the rest being saved for the extraction with phenol. Acid-soluble and lipid compounds were removed by treating the material consecutively with cold 10 % trichloroacetic acid (twice), 80 % ethanol, ethanol saturated with sodium acetate, ethanol, acetone, ethanol–chloroform (3:1), ethanol–ether (3:1) and ether. Dried powder was hydrolysed in 0.5 *N* KOH (1 ml for 100 mg of dry material) for 20 h at 37°, ice-chilled and acidified with 10 *N* perchloric acid. The supernatant was combined with a small volume of cold 0.2 *N* perchloric acid wash, and its pH adjusted to 8.5 with 10 *N* KOH. The centrifuged supernatant contained total RNA. The acid-precipitate was dissolved in 0.1 *N* NaOH and acidified with *N* HCl. The precipitate was then neutralized and washed successively with a mixture of water and ethanol saturated with sodium acetate (1:4), and ethanol saturated with sodium acetate, then twice with ethanol and twice with ether, and dried. The dried powder served as the total DNA fraction but still contained a large amount of protein.

Extraction of RNA and DNA with hot dodecyl sulphate solution. Sometimes this method was employed instead of the foregoing fractionation procedure. Details were given in a previous communication[14]. After the acid-precipitated DNA was separated from RNA, it was washed and neutralized as described previously[16].

Extraction of RNA with phenol. This was made essentially according to KIRBY[10]. The homogenate or cell suspension (even after coagulation of cells during the incubation[14], no homogenizing was necessary) was mixed with 1/20 volume of 2.5 % solution of carrier $Na_2HPO_4 \cdot 12H_2O$ and an equal volume of 90 % phenol, and stirred for 1 h. The mixture was centrifuged at 1,300 *g* for 30 min. The aqueous phase was removed and the phenol phase was washed once with an equal volume of distilled water and centrifuged as above. The third wash was unnecessary because it usually gave no appreciable precipitate upon adding ethanol. The combined aqueous phase was made 1 % with respect to sodium acetate, and RNA was precipitated with 2 volumes of ethanol. The collected precipitate was dissolved in water, sodium acetate was dissolved to 1 %, and RNA was again precipitated with ethanol. It was taken up in a small volume of water after centrifuging and dialysed against cold water for 48 h, or for 4 h under constant stirring, with frequent changes of water. The RNA was again precipitated from the dialysed solution with sodium acetate and ethanol, washed twice with ethanol and ether and dried. A portion of RNA was then hydrolysed with alkali as in the case of total RNA and the remainder digested with phosphodiesterase. This RNA fraction will be called "phenol-released RNA". The expression "phenol-extracted RNA" might be misleading, because this RNA is extracted in the water layer by the action of aqueous phenol, while the phenol layer contains another RNA fraction.

Recovery of RNA from phenol extraction residue. The phenol phase was treated with 4 vol. of ethanol, and, if necessary, 1 vol. of ether, to precipitate the protein completely, and the precipitate was washed once with ethanol. Then it was processed as in the case of total RNA or RNA, extracted with hot dodecyl sulphate, starting with the extraction with cold 10 % trichloroacetic acid, and RNA was recovered by the alkaline hydrolysis as 2,′3′-mononucleotides. This RNA fraction will be called "residual RNA". DNA may be recovered from the acid-precipitated residue as described above.

Enzymic digestion. Phenol-released RNA prepared from incubated materials usually contained some DNA. If the amount of DNA, determined by diphenylamine reaction, was not negligible, phenol-released RNA was first digested with pancreatic DNase (Worthington Biochemical Corp.): 10 mg of RNA specimen and 0.3 mg of crystalline DNase I were dissolved in 3 ml of water and 0.8 ml of 0.1 M $MgSO_4$, the pH adjusted with 0.1 N NaOH to 7.0 and the mixture incubated at 37° for 2 h. Then 0.1 g of sodium acetate was dissolved and 6 ml of ethanol was added to precipitate RNA, which was dissolved and reprecipitated once more and washed twice with ethanol. It was then digested with 1 unit[17] of snake venom phosphodiesterase (a preparation from *Trimeresurus flaviviridis* venom, supplied by Dr. S. IWANAGA, Department of Pharmaceutics, Kyoto University) in 0.9 ml of 0.1 M glycine buffer (pH 9.0), 0.4 ml of 0.36 M $MgSO_4$–0.03 % sodium merthiolate and 2.8 ml of water. After 24 h-incubation at 37° 0.5 unit of phosphodiesterase was added and the mixture incubated for another 24 h. It was then centrifuged, and the supernatant was saved as 5′-mononucleotides.

For the digestion of DNA, either the crude DNA extracted with dodecyl sulphate or the total DNA fraction still containing much protein was used. In both cases a quantitative recovery of the deoxynucleotides was obtained. Samples containing 10 mg of DNA were dissolved or suspended in 3 ml of water, and mixed with 0.8 ml of 0.1 M $MgSO_4$ and 0.2 ml of crystalline DNase I solution containing 0.4 mg of the enzyme. During the incubation at 37° for 1 h, the pH was kept at 7.0 with 0.1 N NaOH. Then 1 ml of 0.1 M $MgSO_4$ and 0.2 unit of phosphodiesterase were added, pH adjusted to 9.0 with 0.1 *N* NaOH, and the incubation continued for 2 h during which the pH was kept at 9.0 with 0.1 *N* NaOH. When digestion was completed, the centrifuged supernatant contained 5′-deoxynucleotides.

Ion-exchange chromatography. Dowex-1 (X8, 200–400 mesh)-formate was employed. For 2′,3′- or 5′-ribonucleotides (160–660 μg P), a 0.28 cm² × 20–22 cm column was connected with a concave gradient elution system[18] with 200 ml of water in the mixer (diameter of 6.5 cm) and 100 ml of 4 *N* formic acid in the reservoir (diameter of 4.5 cm). It was usually necessary to add finally 50 ml of 4 *N* formic acid to complete the elution of 2′,3′- or 5′-uridylic acid. Deoxynucleotides (about 600μg P) were charged on a 0.28 cm² × 30 cm column of the same resin, and eluted in a concave gradient elution system with 300 ml of water in the mixer (8 cm) and 200 ml of 2 *M* ammonium formate (pH 4.25), as recommended by CANELLAKIS AND MANTSAVINOS[19], in the reservoir (6.5 cm). The nucleotide composition of DNA and RNA was determined with separated 5′-deoxynucleotides and 2′,3′-ribonucleotides, respectively, by ultraviolet spectrophotometry[2, 20].

Specific activity measurement. After the determination of total optical density at desired wavelengths, the solutions of monocleotides were adjusted to pH 3 either with ammonia or with formic acid, and adsorbed on a small column of charcoal (Sirasagi for chromatography, Takeda Pharmaceutical Industries, Osaka, Japan; preliminarily treated with 0.01 *M* ethylenediaminetetraacetate). For this, 100 mg of charcoal for 200 μg or less of nucleotide P was packed in a glass tube with a diameter of 0.4 cm, and the mononucleotides were charged on it. The column was then washed 3 times with 10 ml each of water and eluted with 2 ml of 0.3% NH_4OH in 50% ethanol. A portion of the eluate was diluted with 0.2 *N* NH_4OH for the ultraviolet spectrophotometry in alkaline solution from which the phosphorus content was calculated. Other portions were plated on watch glasses for the radioactivity measurement conducted as before[21] in an end-window type Geiger–Müller counter. The specific activity was expressed in counts/min/μg P. The specific activity of the nucleic acid was calculated as the weighted mean of those of the component nucleotides, using the nucleotide composition determined with the same material. The specific activity of respective nucleic acid fractions was sometimes measured also directly as in a previous report[14].

RESULTS

Specific activity determination of nucleic acids

As shown in Tables I and II, specific activities of phenol-released RNA as calculated from those of 2′,3′-nucleotides and of 5′-nucleotides agreed very well in most cases, especially with lymphatic cells, suggesting that the determination of the specific activities of nucleotides was not affected by radioactive contamination. However, the whole RNA preparation, obtained by phenol extraction in the *in vitro* experiments, even after a prolonged dialysis, usually gave a much higher specific activity owing to the serious contamination, just like RNA fraction of the Schmidt–Thannhauser method (Table I). The hot dodecyl sulphate extraction of nucleic acids followed by an extensive purification thereof as carried out in SIBATANI's procedure[14] seems to eliminate the radioactive contamination from the nucleic acid preparation quite effectively, as is evidenced by the good agreement of the specific activities determined directly with those calculated from the measurements on isolated nucleotides (Table I).

TABLE I

SPECIFIC ACTIVITY OF NUCLEIC ACIDS OF RABBIT LYMPHATIC CELLS INCORPORATING $^{32}PO_4$ *in vitro* OR *in vivo*

Weighted means of the specific activities of component nucleotides are given. Figures in parentheses are the specific activity of respective crude nucleic acid fractions as determined prior to the resolution into nucleotides.

Nucleic acid	*Specific activity (counts/min/μg P)*					
	Schmidt–Thannhauser method		*Dodecyl sulphate extraction*		*Phenol-released fraction*	
	DNA	*RNA*	*DNA*	*RNA*	*RNA*	
Nucleotides analysed	5′	2′,3′	5′	2′,3′	2′,3′	5′
In vitro, 1 h			33.4* (16.3)*	87.3 (89.5)	24.9 (97.0)	26.6
In vitro, 1 h	8.2 (14.1)	54.2 (156)			10.1** (101)	8.34
In vitro, 2 h			9.63 (9.55)	38.2 (40.0)	12.1 (48.9)	12.9
In vitro, 2 h	23.5 (24.0)	93.6 (168)			33.6 (133)	33.6
In vivo, 2 h	4.04	9.53			4.36	4.26

* The reason for the discrepancy is not clear. A radioactive contamination of some of the deoxynucleotides is unlikely, since the pattern of specific activities of 4 nucleotides were quite similar to that obtained in other experiments[22].

** Probably a little too high because of a radioactive contamination of 2′,3′-uridylic acid.

TABLE II

SPECIFIC ACTIVITY OF NUCLEIC ACIDS OF RAT LIVER SLICES INCUBATED WITH $^{32}PO_4$ FOR 2 H

Nucleic acid	*Specific activity (counts/min/μg P)*				
	Schmidt–Thannhauser method		*Phenol-released fraction*		*Residual fraction*
	DNA	*RNA*	*RNA*		*RNA*
Nucleotides analysed	5′	2′,3′	2′,3′	5′	2′,3′
Normal liver (24 h)	0.34*	5.55**	—	0.58	37.6**
Regenerating liver (24 h)	0.50	5.45***	2.65**	2.12	20.2**
Regenerating liver§ (30 h)	19.8	28.7**	10.7	12.5	91.9§§

* Extracted with hot dodecyl sulphate from alkaline hydrolysis residue. The specific activity was determined directly without separating nucleotides.

** Probably a little too high because of a radioactive contamination of 2′,3′-uridylic acid.

*** Average of the specific activities of 3 nucleotides except adenylic acid, which was lost.

§ Incubated with 5 μC/ml of $^{32}PO_4$. In two other experiments 2 μC/ml of $^{32}PO_4$ were used.

§§ Average of the specific activities of adenylic and cytidylic acids, the other two nucleotides being lost.

It has been found, however, in recent unpublished experiments that 2′,3′-uridylic acid (but not 5′-uridylic acid) as obtained by the present method may occasionally be subjected to some radioactive contamination, especially in the liver. But this may not have seriously distorted the overall picture reported in this paper, because the specific activities of RNA calculated from those of 2′,3′- and of 5′-nucleotides showed a fairly good agreement, even in cases where an appreciable contamination of 2′,3′-uridylic acid was suspected (Tables I and II).

Fractionation of RNA's with different specific activities

The principal finding of the present study is a fractionation of metabolically distinct RNA's, which can be achieved by directly treating the cellular material with phenol, without preliminary isolation of RNA. As is listed in Tables I and II, the specific acitvity of phenol-released RNA was always much lower than that of RNA obtained by the quantitative Schmidt–Thannhauser method or by SIBATANI's purification procedure[14] involving hot dodecyl sulphate extraction. Moreover, the phenol-released RNA had a specific activity sufficiently close to that of DNA in both lymphatic cells and normal liver. This is especially remarkable when one notices that DNA in lymphatic cells took up much radiophosphate, while that in normal liver incorporated very little of it, reflecting the difference in their proliferative activity. This finding led us to a search for another RNA fraction (residual RNA) that might remain attached to proteins in the phenol layer and incorporate ^{32}P-orthophosphate at a considerably high rate. As was expected, a residual RNA fraction with such properties was actually recovered from both rat liver (Table II) and lymphatic cells (*cf.* SIBATANI *et al.*[11]).

Specific activities of individual nucleotides derived from different nucleic acid fractions are presented in a separate paper[22].

Nucleotide composition of nucleic acid fractions

Table III compiles some typical examples of nucleotide composition of various nucleic acid fractions as determined in individual experiments. They served in respective experiments as the basis for calculating the specific activities of nucleic acids from those of the isolated nucleotides. In the present study the nucleotide composition of total DNA was determined without preliminary purification; the values obtained with DNA of lymphatic cells and the rat liver agreed fairly well with the published data for mammalian DNA.

TABLE III

NUCLEOTIDE COMPOSITION OF DNA AND RNA OF LYMPHATIC CELLS ISOLATED FROM RABBIT APPENDIX AND PEYER'S PATCH AND OF RAT LIVER

Material	*Nucleic acid fraction*	*Molar ratio (Adenine-containing nucleotide = 10). Nucleotide containing:*				E_{260} *recovery (%)*
		Guanine	*Cytosine*	*Uracil*	*Thymine*	
Lymphatic cells	Total DNA	7.56	7.70		10.4	90.7
Normal liver	Total DNA	7.54	7.74		10.9	100
Lymphatic cells	Total RNA	18.1	15.3	9.9		90.3
	Total RNA*	17.5	15.3	10.1		96.5
Normal liver	Total RNA	18.0	16.7	9.0		88.2
Lymphatic cells	Dodecyl sulphate RNA	18.7	16.0	11.5		102
Lymphatic cells	Phenol-released RNA	20.2	16.6	12.5		90.2
	Phenol-released RNA	19.9	16.7	9.7		98.3
	Phenol-released RNA*	20.2	16.2	10.6		101
Normal liver	Phenol-released RNA	19.8	15.4	11.1		98.1
Normal liver	Residual RNA	14.5	15.3	9.0		79.8
Regenerating liver (24 h)	Residual RNA	15.0	15.8	7.2		78.0

* Analysed with freshly prepared cells. In other cases cells or tissues were incubated *in vitro* for 1–2 h.

The estimation of the amount of uridylic acid may not be so accurate as that of other nucleotides in the present method, owing to the higher background optical density of Dowex-1 eluates containing this nucleotide. In fact, the uridylic acid content in RNA was not highly reproducible in repeated determinations.

As shown in Table III, phenol-released RNA had a higher guanylic acid content (and probably higher cytidylic acid content in lymphatic cells) than total RNA. As can be expected, the residual RNA of the rat liver showed a markedly low guanylic acid content. The guanylic acid content of dodecyl sulphate-extracted RNA of lymphatic cells was also not so high as that of phenol-released RNA.

RNA fractions in the regenerating rat liver

Since the specific activity of phenol-released RNA agreed rather well with that of DNA in both proliferating lymphatic cells and non-proliferating rat liver, it was thought to be of interest to examine the situation in the regenerating rat liver in which a wave of activity of DNA synthesis is reported to occur around 24 h post-operatively[15, 23, 24]. However, in our present material slices of 24-h regenerating livers showed a very slight incorporation of ^{32}P-orthophosphate into DNA, so that we also employed 30-h regenerating livers. It is seen from Table II that in the 24-h regenerating liver, the specific activity of phenol-released RNA was much higher than that of DNA but in the 30-h regenerating liver the situation was reversed. If such an observation can be confirmed, it will imply that in regenerating liver a burst of synthesis of phenol-released RNA precedes the onset of DNA synthesis and its rate also declines earlier than that of DNA synthesis. Indeed, the active RNA synthesis in regenerating liver seems to start several hours ahead of the DNA synthesis[15] and thus may correspond to the observed earlier enhancement of ^{32}P-incorporation into phenol-released RNA.

Proportion of phenol-released and residual RNA

Although in the present study a careful estimation of the proportion of two RNA fractions was not attempted, the relative yield of the two fractions was well paralleled by the proportion of the two fractions as estimated from the specific activities of total, phenol-released and residual RNA of the rat liver. Thus RNA of this tissue comprised approximately 85 % phenol-released and 15 % residual RNA fraction. In rabbit lymphatic cells the proportion of phenol-released RNA was much lower, occupying about 70 % of the total RNA, and in this case also this value was consistent with the specific activities of the RNA fractions[11]. The guanylic acid content of different RNA fractions (Table III) was also in harmony with such an estimate.

DISCUSSION

In this paper, the phenol treatment of cellular material has been shown to effect a fractionation of two metabolically distinct forms of RNA; a fraction released by the action of phenol into the aqueous phase had a low specific activity and a high guanylic acid content, while the residual fraction remaining in the phenol phase together with proteins showed a high specific activity and a low guanylic acid content. In this study both fractions were obtained and analysed in the liver. But in lymphatic cells the presence of the residual fraction could be deduced only from the specific activities and nucleotide compositions of the total and phenol-released RNA, and the actual demonstration of its existence was later realised in this laboratory[11]. The whole appendix and liver of rabbits likewise gave the two fractions as expected[12]. The study was further extended by one of the authors (K.Y.) to the Ehrlich ascites carcinoma in the Department of Biology, Kyushu University, Fukuoka, and this material also yielded the two fractions of RNA with properties in line with the above generalization[13].

These findings thus clearly indicate that RNA of a variety of animal cells can be fractionated by phenol treatment into two components whose properties are quite alike among different types of tissues. It follows that the phenol method of KIRBY[10] may fail, under certain conditions, to extract a representative sample of RNA from many types of material, thereby missing the metabolically most active fraction. The extraction with hot dodecyl sulphate solution[14], on the other hand, apparently yields much more satisfactory preparations of RNA in this respect.

Now it may be noted that residual RNA, by its high activity in incorporating labelled precursors and low guanylic acid content, resembles a fraction of RNA contained in the nuclear material insoluble in phosphate buffer[2,4,5]. There is evidence indicating that the latter RNA is probably of nucleolar origin[25]. One might therefore be inclined to suspect that the phenol treatment simply released the bulk of cellular RNA without touching the "nucleolar" RNA, which was then recovered as the residual RNA in our procedure. Indeed, the proportion of residual RNA was higher in lymphatic cells with scanty cytoplasm than in the liver with abundant cytoplasm. However, in Ehrlich ascites carcinoma cells with relatively small cytoplasm the proportion of residual RNA was remarkably low[13].

Furthermore, the fact that phenol-released RNA had a specific activity very close to that of DNA strongly suggests that this RNA fraction may not simply be identical with the "non-nucleolar" RNA. HOTTA AND OSAWA[26] reported that in proliferating tissues such as the thymus or appendix, the bulk of cytoplasmic RNA and also the fraction of nuclear RNA extracted in phosphate buffer took up [^{32}P]-orthophosphate at almost the same rate as DNA. It is also widely accepted, however, that in many tissues including the liver, the isotope uptake of cytoplasmic RNA is appreciably higher than that of DNA as revealed with various labelled precursors[27]. Now it would seem more likely that some of the subcellular components may contain both phenol-released and residual RNA fractions. The results so far obtained in this laboratory indicate that such is the case at least with rabbit appendix (ref. 12 and KIMURA AND SIBATANI, to be published). Thus, the phenol method may represent a novel approach to the fractionation of RNA in animal cells and would provide us with information concerning some of the hitherto unexplored properties of cellular RNA.

Under these circumstances, it would be pertinent to assume the occurrence in animal cells of two main classes of RNA: class I, with low ^{32}P uptake and high guanylic acid content, may be synthesized at about the same rate as DNA in the steady state (therefore duplicated only once during the mitotic cycle) and hence metabolically rather inert like DNA[15,21,28], and class II, with high guanylic acid content, is on the other hand turned over at an exceedingly high rate. These two classes of RNA can be recovered as phenol-released and residual RNA fractions, respectively, by treatment of fresh cellular material with aqueous phenol under appropriate conditions, probably owing to the difference in the mode of linkage to proteins. By correlating the metabolic activity of RNA with the mode of linkage between RNA and protein, we may begin to understand the mechanism that controls the rate of RNA turnover in living cells. Further implications of the hypothesis of two classes of RNA will be dealt with in later communications of this series.

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SOME ASPECTS OF DIFFERENTIAL LABELLING OF INDIVIDUAL MONONUCLEOTIDES DERIVED FROM NUCLEIC ACIDS OF ANIMAL CELLS INCORPORATING [^{32}P]ORTHOPHOSPHATE

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SUMMARY

Specific activities were determined of individual 2',3'-nucleotides derived from various RNA fractions and 5'-nucleotides derived from DNA and phenol-released RNA in rabbit lymphatic cells and rat liver slices incorporating [^{32}P]orthophosphate for 1–2 hours *in vivo* or *in vitro*. The pattern of the specific activity of these nucleotides showed a marked difference between the lymphatic cells and the liver in several respects. In lymphatic cells, 5'-AMP derived from phenol-released RNA had a much lower specific activity than the other 5'-nucleotides, while in the liver 5'-AMP showed the highest specific activity among the 5'-nucleotides. In lymphatic cells, the incorporation of radiophosphate into purine 5'-nucleotides of DNA was much lower than that into pyrimidine 5'-nucleotides *in vitro*, but the experiments *in vivo* did not result in such an uneven incorporation. Neither phenol-released RNA of lymphatic cells nor DNA of rat liver gave a similar uneven incorporation into purine and pyrimidine 5'-nucleotides *in vitro*. The incorporation pattern into 2',3'-nucleotides was somewhat different between total and phenol-released RNA in lymphatic cells. Again no such difference was noted in the liver. A close agreement of the total acitvity of four 2',3'-nucleotides of RNA was noted on some occasions, but apparently this was not a general phenomenon. The significance of these findings was discussed briefly.

INTRODUCTION

In a separate paper[1] we have reported the fractionation of RNA of some animal cells with phenol; the specific activity of various nucleic acid fractions was determined as the weighted mean of those of the individual mononucleotides derived from each nucleic acid. A closer examination of the pattern of specific activities of various mono-

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; AMP, GMP, CMP, UMP, adenylic, guanylic, cytidylic and uridylic acid, respectively, 5'- and 2',3'-isomers being distinguished by the prefix like 5'-AMP or 2',3'-GMP; dAMP, dGMP, dCMP, dTMP, deoxyadenylic, deoxyguanylic, deoxycytidylic and deoxythymidylic acid, respectively, all being 5'-isomers.

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nucleotides has now indicated several points of interest, which will be briefly reported in this paper.

EXPERIMENTAL

Since all the results were obtained from the same experiments described in a separate paper[1], the details of the experimental procedures need not be recapitulated here.

RESULTS AND DISCUSSION

We determined the specific activities of individual 5′-nucleotides derived from DNA and phenol-released RNA, and 2′3′-nucleotides derived from various RNA fractions of lymphatic cells of rabbit appendix and rat liver incorporating [^{32}P]orthophosphate for 1–2 h *in vitro* or *in vivo*. The results are compiled in Tables I to III.

A remarkable difference was found between the lymphatic cells and the liver in the pattern of the specific activities of 2′,3′- and 5′-nucleotides of phenol-released RNA (Table I). In the lymphatic cell the specific activity of 5′-AMP was consistently and significantly lower than that of 2′,3′-AMP. It was also appreciably lower than any of the specific activities of other 5′- or 2′,3-nucleotides. By contrast, the opposite was the case with the regenerating liver: the specific activity of 5′-AMP derived from phenol-released RNA was significantoy higher than that of other 5′-nucleotides and of most of the 2′,3′-nucleotides, including 2′,3′-AMP, derived therefrom. As regards the specific activities of 2′,3′-nucleotides, 2′,3′-AMP did not stand out among others

TABLE I

COMPARISON OF THE SPECIFIC ACTIVITIES OF 2′,3′- AND 5′-ISOMERS OF THE NUCLEOTIDES DERIVED FROM PHENOL-RELEASED RNA OF RABBIT LYMPHATIC CELLS AND RAT LIVER SLICES INCORPORATING $^{32}PO_4$ *in vitro* OR *in vivo*

Material	*Condition*	*Isomer*	*Specific activity (counts/min/μg P)*			
			AMP	*GMP*	*CMP*	*UMP*
Lymphatic cells	*In vitro*, 1 h	2′,3′	20.9	24.4	25.5	28.6
		5′	9.4	25.2	35.6	29.4
	In vitro, 1 h	2′,3′	9.93	8.34	7.56	16.5*
		5′	6.72	9.18	7.67	8.95
	In vitro, 2 h	2′,3′	11.8	10.7	13.4	12.9
		5′	8.45	12.7	15.2	14.2
	In vitro, 2 h	2′,3′	36.3	31.2	35.5	40.1
		5′	21.9	34.0	25.9	39.1
	In vivo, 2 h	2′,3′	4.54	4.56	4.37	3.78
		5′	3.54	4.92	4.02	5.35
Normal liver	*In vitro*, 2 h	2′,3′	—	0.37	—	0.38
		5′	1.04	0.27	0.81	0.48
Regenerating liver	24 h after operation; *in vitro*, 2 h	2′,3′	1.95	1.49	2.46	5.90*
		5′	3.36	1.35	2.51	1.56
	30 h after operation; *in vitro*, 2 h	2′,3′	14.0	8.90	9.50	14.7
		5′	26.9	9.72	9.57	7.99

* Probably contaminated by some extraneous radioactive material.

TABLE II

COMPARISON OF RELATIVE SPECIFIC ACTIVITIES OF INDIVIDUAL 2′,3′-NUCLEOTIDES OF DIFFERENT RNA FRACTIONS OF RABBIT LYMPHATIC CELLS AND RAT LIVER SLICES INCORPORATING $^{32}PO_4$ *in vivo* OR *in vitro*

Material	*Condition*	*Fraction of RNA*	*Relative specific activity (AMP = 1.0)*		
			GMP	*CMP*	*UMP*
Lymphatic cells	*In vitro*, 1 h	Dodecyl sulphate	1.01	0.83	1.19
		Phenol-released	1.17	1.22	1.42
	In vitro 1 h	Total	0.65	0.56	0.49
		Phenol-released	0.84	0.76	1.66*
	In vitro, 2 h	Dodecyl sulphate	0.66	0.72	0.43
		Phenol-released	0.91	1.14	1.09
	In vitro, 2 h	Total	0.65	0.73	1.12
		Phenol-released	0.86	0.98	1.10
	In vivo, 2 h	Total	0.80	0.80	0.99
		Phenol-released	1.00	0.96	0.83
Normal liver	*In vitro*, 2 h	Total	0.53	0.82	1.62*
		Residual	0.51	0.62	3.34*
Regenerating liver	24 h after operation; *in vitro*, 24 h	Total**	(0.63)	(0.84)	(1.30)*
		Phenol-released	0.77	1.26	3.02*
		Residual	0.63	0.79	1.85*
	30 h after operation; *in vitro*, 2 h	Total	0.70	0.74	1.50*
		Phenol-released	0.64	0.68	1.05*

* Probably contaminated by extraneous radioactive material and not reliable.
** Owing to the loss of AMP, the value of GMP was arbitrarily taken as 0.63.

TABLE III

RELATIVE SPECIFIC ACTIVITIES OF 5′-NUCLEOTIDES OF DNA AND PHENOL-RELEASED RNA OF RABBIT LYMPHATIC CELLS AND RAT LIVER SLICES INCORPORATING $^{32}PO_4$ *in vitro* OR *in vivo*

Material	*Condition*	*Relative specific activity (AMP or dAMP = 1.0)*					
		DNA			*RNA*		
		dGMP	*dCMP*	*dTMP*	*GMP*	*CMP*	*UMP*
Lymphatic cells	*In vitro*, 1 h	1.54*	4.51*	3.23*	2.37	3.34	2.76
	In vitro, 1 h	1.76	5.35	5.48	1.37	1.14	1.33
	In vitro, 2 h	1.61*	3.30*	3.57*	1.50	1.80	1.68
	In vitro, 2 h	1.79	2.95	3.34	1.55	1.64	1.78
	In vivo, 2 h	1.26	1.32	1.60	1.39	1.27	1.51
Normal liver	*In vitro*, 2 h	—	—	—	0.26	0.78	0.48
Regenerating liver	24 h after operation; *in vitro*, 2 h	1.19	0.87	1.02	0.40	0.75	0.46
	30 h after operation; *in vitro*, 2 h	0.74	1.47	1.48	0.36	0.36	0.30

* DNA was extracted with hot dodecyl sulphate; in other experiments they were obtained by Schmidt–Thannhauser fractionation.

in both materials. Corresponding to the finding of a lower specific activity of 5'-AMP than of 2',3'-AMP in RNA of the lymphatic cell, the specific activities of 5'-GMP and 5'-CMP of the same RNA were usually found a little higher than, though sometimes close to, the corresponding 2',3'-isomers. No definite trend has so far been detected in the specific activities of these nucleotides in the liver.

The results obtained with phenol-released RNA of the rat liver confirm those reported by SHIGEURA AND CHARGAFF[2] of RNA's of several cytoplasmic components in the rat liver. Incidentally, the agreement between the specific activities of RNA as estimated with 2',3'-nucleotides and those with 5'-nucleotides was much more satisfactory in our experiments than in those of the American workers, who noted that the specific activity of RNA calculated with 5'-nucleotides was invariably lower than that with 2',3'-nucleotides. The higher specific activity of 5'-AMP as compared with other 5'-nucleotides derived from the same RNA was also noted with RNA's of some plant viruses by STAEHELIN[3]. He concluded that the plant virus RNA was probably synthesized from 5'-nucleotide intermediates, because the specific activities of 2',3'-nucleotides were very uniform, while those of 5'-nucleotides were definitely diverse. Such a trend was noted, although less distinct, also with RNA's of the rat liver and rabbit lymphatic cells in SHIGEURA AND CHARGAFF's and our own studies. STAEHELIN's view is in line with the recent findings[4,5,6,7,12] about the enzymic syntheses of polynucleotides with 5'-nucleotides as substrates.

If such a view is correct, the situation encountered in the lymphatic cell may be explained by a relatively large pool size of acid-soluble adenine 5'-nucleotides which would dilute out the ^{32}P to be incorporated into the 5'-AMP moiety of RNA. Obviously such an argument does not apply in the case of the liver. Here one must assume that ^{32}P is introduced at a remarkably high rate into the proximal phosphate of the acid-soluble adenine 5'-nucleotides and/or that there is an extensive turnover of the 5'-AMP moiety in at least some of the RNA's contained in phenol-released RNA fraction of the liver cytoplasm. The latter point recalls the incorporation of AMP into the supernatant RNA involved in amino acid transfer[8].

When the specific activities of 2',3'-GMP and CMP relative to that of 2',3'-AMP were compared between total or dodecyl sulphate-extracted RNA on the one hand and phenol-released RNA on the other hand, those of the latter were noted to be consistently higher in the lymphatic cell, but again such a trend could not be noticed in the liver (Table II). It could not be determined whether specific activities of 2',3'-UMP might show any definite trend, owing to its lower reproducibility probably caused by some radioactive contamination. The observed difference among RNA fractions of lymphatic cells may be a reflection of the difference in nucleotide sequence between phenol-released and residual RNA which can be fractionated with phenol[1]. For such an argument to be convincing, however, we must first know whether 5'-AMP derived from the residual RNA has a much lower specific activity than that of other 5'-nucleotides, as in the case of phenol-released RNA. Such an argument does not apply in the case of the liver, in which no consistent difference was found in the specific activities of 2',3'-nucleotides among various RNA fractions.

As regards DNA, ORD AND STOCKEN[9] demonstrated that in thymocytes of rats the *in vitro* incorporation of [^{32}P]orthophosphate into purine 5'-deoxynucleotides was about one tenth as small as that into pyrimidine 5'-deoxynucleotides, while the incorporation *in vivo* into both types of deoxynucleotides took place apparently to a comparable extent. Such an observation was confirmed by our experiments on rabbit lymphatic cells, although the difference between purine and pyrimidine nucleotides was much less pronounced. As shown in Table III, in most of the *in vitro* experiments the specific activities of the dCMP and dTMP were more than three times that of dAMP and more than twice that of dGMP. Therefore, the specific activity of dGMP was more than 1.5 times that of dAMP.

Such a pattern of the specific activities of individual 5′-deoxynucleotides *in vitro* was highly reproducible. *In vivo* also, the specific activity of dAMP was lowest, and pyrimidine deoxynucleotides had higher specific activities than purine deoxynucleotides. However, the difference was rather slight and not so remarkable as that *in vitro*.

ORD AND STOCKEN[9] tried to explain the observed difference *in vitro* by postulating that the synthesis of purine deoxynucleotides in thymocytes was inhibited under *in vitro* conditions while that of pyrimidine deoxynucleotides was not depressed. Their inference was based on the observation of SMELLIE *et al.*[10] that under *in vitro* conditions the *de novo* synthesis of the purines in the thymus was severely affected, in contrast to the unimpaired synthesis of thymine, as revealed by the incorporation of [^{14}C]-formate. If ORD AND STOCKEN's idea were correct, it would follow that the same inhibition pattern of the ^{32}P incorporation would be observed also with 5′-nucleotides derived from RNA. As listed in Table III, the specific activity of 5′-GMP of the phenol-released RNA was not inferior to those of pyrimidine 5′-nucleotides. As mentioned above, however, the specific activity of 5′-AMP of this RNA fraction was always lower than that of other 5′-nucleotides. But in this case no significant difference emerged between *in vivo* and *in vitro* conditions, and the difference between 5′-AMP and other 5′-nucleotides was, with one exception, of the same magnitude as that observed with deoxyribonucleotides *in vivo*. It is therefore difficult to accept the idea proposed by ORD AND STOCKEN to explain the uneven incorporation of ^{32}P into DNA nucleotides *in vitro*. It would be no less difficult, however, to propose another explanation for it. Whether this non-uniform incorporation means some turnover of pyrimidine nucleotide moiety in DNA *in vitro* cannot be determined at the moment. However, it should be mentioned that the specific activity of DNA was usually very close to that of phenol-released RNA both *in vivo* and *in vitro*[1].

Further complexity about this problem was disclosed, when one compared the above results with those of the rat liver, where the situation was entirely different. In DNA, the difference in the specific activity between purine and pyrimidine nucleotides was not very significant; even if the incorporation into pyrimidine deoxynucleotides was higher than that into purine deoxynucleotides, the difference was rather slight. It was thus revealed that the uneven incorporation of ^{32}P into DNA *in vitro* was not a general phenomenon, and as was suggested by ORD AND STOCKEN[9], it is certainly reminiscent of the altered incorporation pattern observed with [^{14}C]-formate *in vitro* in certain proliferating tissues[10].

The last point to be raised concerns the cases of non-uniform labelling of 2′,3′-nucleotides derived from RNA. SMELLIE[11] has already noted that generally an inverse relationship exists between the specific activity of ^{32}P and the molar composition of individual 2′,3′-nucleotides of RNA in animal cells. Then it follows that the total activity of 2′,3′-nucleotides would be very similar. As shown in Table IV, lines 1 and 5, both total and phenol-released RNA may show a uniform distribution of total

TABLE IV

SOME EXAMPLES OF THE DISTRIBUTION OF RADIOACTIVITY AMONG 2′,3′-NUCLEOTIDES OF RNA FROM RABBIT LYMPHATIC CELLS OR RAT LIVER INCORPORATING $^{32}PO_4$ *in vitro* OR *in vivo* FOR 2 H

Material	*Nucleic acid*	*Total activity* (% of total)*			
		AMP	*GMP*	*CMP*	*UMP*
Lymphatic cells *in vitro*	Total RNA	22.8	26.6	25.4	25.2
	Phenol-released RNA	17.2	30.1	28.4	24.3
Lymphatic cells *in vivo*	Total RNA	21.6	30.4	26.4	21.6
	Phenol-released RNA	18.3	37.4	28.4	15.9
Regenerating rat liver *in vitro*	Phenol-released RNA	24.2	25.9	23.9	25.8

* Calculated from the specific activity and proportion of individual mononucleotides for each nucleic acid specimen.

activity among four nucleotides. But apparently there are cases which do not fit in with such a regularity (Table IV, lines 2–4). The uniform total labelling of 2′,3′-nucleotides of RNA is in a marked contrast with the uniform specific activities of 2′,3′-nucleotides as was shown in plant virus RNA[3]. If the latter observation was due to the homogeneity of RNA while the former was caused by the heterogeneity thereof, the discrepancy might be explained. The results are suggestive but the interpretation is difficult. If, as is generally believed[3, 12], RNA is synthesized with 5′-nucleotide intermediates, this does not exclude the possibility that the good agreement of the total activities of 2′,3′-nucleotides was fortuitous.

The observations discussed are all of indirect nature in respect of the determination of the nucleotide sequence or the understanding of the mechanism of nucleic acid biosynthesis, and more data on extensively fractionated nucleic acids will have to be accumulated until some insight into such problems can be obtained by an approach along these lines.

ACKNOWLEDGEMENTS

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SYNTHESIS OF LIPIDS BY DIFFERENT HUMAN BLOOD CELL TYPES

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SUMMARY

A comparison has been made of the incorporation *in vitro* of [^{14}C]acetate into the lipids of leucocytes and red cells of human blood. In the course of incubation of whole human blood at 35° for periods up to 5 h, the leucocytes were responsible for all of the detectable incorporation into the unsaponifiable lipid and most of that into the phospholipid fatty acids. There was considerable incorporation into the neutral saponifiable lipid fatty acids of both leucocytes and red cells.

INTRODUCTION

It has been known for some years that mammalian blood cells can synthesize lipids from acetate and phosphate. ALTMAN, WHATMAN AND SALOMON[1] and ALTMAN[2] found that radioactive acetate is incorporated into the lipids of rabbit erythrocyte stroma. Considerable *in vitro* incorporation of acetate into the fatty acids of human blood cells has been reported by JAMES *et al.*[3]. It has been shown also that acetate and phosphate are simultaneously incorporated *in vitro* into human blood cell phospholipids[4].

The individual contributions to lipid synthesis of the several types of blood cell are still uncertain. Recently O'DONNELL *et al.*[5] reported considerable *in vitro* incorporation of radioactive acetate into the lipids of rabbit reticulocytes and fowl leucocytes. The leucocytes from normal mammalian blood and thymus tissue showed little or no incorporation, whereas leucocytes of human patients with leukaemia incorporated labelled acetate into lipids. Since lipids are known to be essential for red cell survival, the synthesis of lipids in the different types of blood cell is a problem of general interest and has been investigated again in an attempt to resolve apparent contradictions. The purpose of this paper is to present evidence that normal leucocytes incorporate relatively large amounts of labelled acetate into lipids of all types. Human red cells appear to synthesize some lipids, although it is possible that the major contribution comes from the reticulocytes.

MATERIALS AND METHODS

Freshly drawn blood was incubated in siliconised glass tubes at 35° for periods up to 5 h in acid-citrate-dextrose (0.83 *M* trisodium citrate, 0.35 *M* citric acid and 0.1 *M* glucose; 1.5 ml/10 ml of blood), streptomycin–penicillin mixture (2:1 by weight; 2 mg/10 ml of blood) and sodium ethylenediamine tetra-acetate (0.03 ml of 0.1 % w/v solution/10 ml of whole blood). This mixture was incubated with Na[2-^{14}C]acetate (specific activity, 12.2 μC/mg), Na[1-^{14}C]acetate (specific activity, 32 μC/mg) or ^{32}P labelled carrier free orthophosphate. Sterile procedures were used throughout the incubation. After incubation the blood was centrifuged at 5000 × *g* for 10 min, the plasma was removed and the cells suspended in a 28 % w/v solution of bovine albumin (Armour) in saline. The cells were fractionated by centrifugation at 500 × *g* for 20 min. The uppermost fraction contained a high proportion of leucocytes and the lowermost fraction mature erythrocytes with the least contamination by leucocytes or reticulocytes. The differential centrifugation procedure was repeated with each of the fractions so isolated. The cells were then separated from albumin and added to 20 volumes of ethanol–ether (2:1 by volume). The ethanol–ether extracts were concentrated to dryness and the residue extracted with acetone to remove neutral fat, which was saponified. The residual acetone-insoluble phospholipids were also saponified. The radioactivity of the unsaponifiable fat and the fatty acids of the saponifiable fat and the phospholipids were assayed using an end-window Geiger-Müller counter and corrected for self absorption.

In other experiments cells were fractionated before incubation with radioactive isotopes. The cellular fractions were separated from albumin, washed with saline and resuspended in their own plasma. The radioactive isotopes were then added. Erythrocyte and leucocyte counts were made in counting chambers, and the reticulocyte counts were made on wet films after supravital staining with brilliant cresyl blue. In each case 1000 cells were counted, reducing the random error of the count to ± 3.2 %. Platelets were included in the leucocyte fraction but were not counted separately.

The results of two typical experiments are shown in Tables I and II. In Fig. 1a, 1b and 1c the specific activities of the lipid fractions are plotted against the ratio of leucocytes to red cells. The specific activity of the unsaponifiable lipid varied linearly with this ratio giving a line which passed close to the origin. The specific activity of the phospholipid fatty acid also varied linearly giving a line which intercepted the abscissa. The reason for the intercept is as yet unknown. Possible explanations include the presence of a metabolically inert and evenly distributed leucocyte fraction; or the presence of short chained radioactive fatty acids not extracted in the fractionation procedure. The specific activity of the saponifiable lipid showed some scatter (Fig. 1c).

TABLE I

THE INCORPORATION OF ACETATE INTO THE CELLULAR LIPIDS OF WHOLE BLOOD

30 ml whole blood was incubated with 30 μC sodium [2-^{14}C]acetate for 5 h. The cells were then separated and fractionated.

Fraction	*No. of leucocytes* $\times 10^{-7}$	*No. of red cells* $\times 10^{-10}$	*Radioactivity counts/min*		
			Saponifiable lipid	*Phospholipid*	*Unsaponifiable lipid*
1	10.6	3.94	230	169	55.2
2	6.8	4.61	183	167	41.7
3	7.3	5.83	198	97	46.8
4	5.8	5.90	166	158	32.1

TABLE II

THE INCORPORATION OF ACETATE INTO THE CELLULAR LIPIDS OF WHOLE BLOOD

A mixture (100 ml) of whole blood and acid–citrate–dextrose solution was incubated with 70 µC sodium [1-^{14}C] acetate for 5 h. The cells were then separated and fractionated.

Fraction	*No. of leucocytes* $\times 10^{-7}$	*No. of reticulocytes* $\times 10^{-8}$	*No. of erythrocytes* $\times 10^{-10}$	*Radioactivity counts/min*		
				Saponifiable lipid	*Phospholipid*	*Unsaponifiable lipid*
1	16.4	3.82	6.32	789	385	208.5
2	3.2	49.2	20.3	74.7	92.4	57.5
3	2.6	3.84	64.12	108.1	50.4	25.4

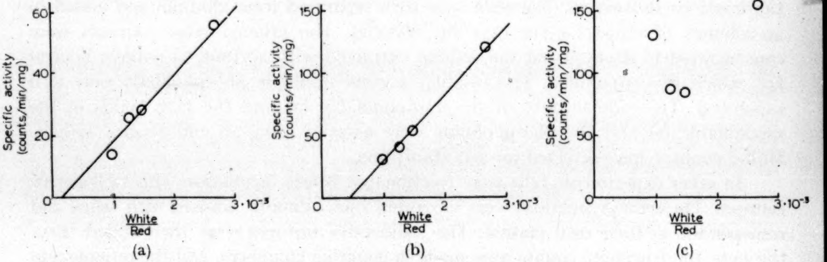


Fig. 1. The incorporation of acetate into the cellular lipids of whole blood. See Table I. (a) Unsaponifiable lipid; (b) Phospholipid; (c) Saponifiable lipid.

This suggests that with respect to this lipid either there is a different metabolic distribution in the white cells or, as seems more likely, the red cells are playing some part.

The results of experiments in which the cell types were fractionated before incubation with radioactive acetate are summarized in Tables III and IV. In these experiments the relative proportions of cell types in the incubation mixture were abnormal. The specific activity of the unsaponifiable lipid in Table III again varied linearly with the ratio of white cells to red cells while the saponifiable and phospholipid

TABLE III

THE INCORPORATION OF ACETATE INTO THE LIPIDS OF CELLULAR FRACTIONS OF BLOOD

The cells from a mixture of whole blood (30 ml) and acid citrate–dextrose solution (7 ml) were separated and fractionated. The cellular fractions were washed and resuspended in their own plasma. Volume of fractions 1, 2, and 3, 10 ml. Volume of fraction 4, 3 ml. Streptomycin penicillin, sodium ethylenediamine tetra-acetate and sodium [2-^{14}C]acetate (1 µC/ml of cell suspension) were added and the mixture incubated for 2.6 h. The cells were then separated, washed with saline and added to ethanol–ether.

Fraction	*No. of leucocytes* $\times 10^{-7}$	*No. of red cells* $\times 10^{-10}$	*Radioactivity counts/min*		
			Saponifiable lipid	*Phospholipid*	*Unsaponifiable lipid*
1	7.75	2.90	21.7	10.5	11.1
2	3.25	6.25	60.4	36.2	3.4
3	2.65	5.40	16.4	36.0	3.6
4	2.00	5.75	21.5	38.2	3.1

TABLE IV

THE INCORPORATION OF ACETATE AND PHOSPHATE INTO THE LIPIDS OF BLOOD

The cells from 100 ml of a mixture of whole blood and acid–citrate–dextrose were fractionated and resuspended in their own plasma. Streptomycin, penicillin and sodium ethylenediamine tetra-acetate were added to each fraction. Volume of each fraction, 10 ml. To fractions 1, 2 and 3, 10 μC sodium [2-^{14}C]acetate was added. To fractions 1a, 2a and 3a, 25 μC of ^{32}P labelled carrier free sodium orthophosphate was added. The fractions were incubated for 5 h and immediately added to ethanol–ether. The extracts from fractions 1, 2 and 3 were treated as described in the text. The extracts of fractions 1a, 2a and 3a were concentrated to dryness. The residue was extracted 3 times with acetone (5 ml) and then 4 times with ethanol (5 ml). The combined ethanol extracts were diluted to 25 ml and 0.1 ml was evaporated to dryness on a planchet.

Fraction	*No. of leucocytes* $\times 10^{-7}$	*No. of reticulocytes* $\times 10^{-8}$	*No. of erythrocytes* $\times 10^{-9}$	*Radioactivity counts/min*		
				Saponifiable lipid	*Phospholipid*	*Unsaponifiable lipid*
1	6.40	2.31	1.87	22.2	875	19.7
2	3.06	5.28	6.07	826	934	14.7
3	0.60	0.496	62.0	42.8	49.2	0.0
1 (a)	6.40	2.31	1.87		$1.47 \cdot 10^4$*	
2 (a)	3.06	5.28	6.07		$1.84 \cdot 10^4$*	
3 (a)	0.60	0.496	62.0		$0.38 \cdot 10^4$*	

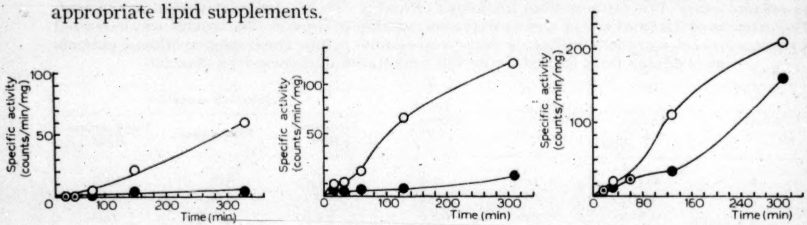
* Radioactivity due to ^{32}P.

fatty acids showed some scatter. In another experiment (Table IV) the plasma was also extracted, and the results include the radioactivity in lipid exchanged by the cells with the plasma during incubation. Fractions 2 and 2(a) which were comparatively rich in reticulocytes showed considerable incorporation of acetate into the saponifiable lipid fatty acids and the phospholipid fatty acids, and of inorganic phosphate into the phospholipid. These experiments show again that most, if not all, of the incorporation of acetate into the unsaponifiable lipid is due to the leucocytes.

These conclusions were confirmed by studies of the progressive incorporations of radioactive acetate into the lipids of leucocytes and red cells over the course of 5 h. In this experiment the cells were fractionated by filtration through ballotini glass beads. Most of the white cells remained on the glass and their numerical ratio to red cells was thereby reduced by a factor of twelve. Radioactivity was incorporated into the unsaponifiable lipid of leucocytes but not into that of red cells (Fig. 2a). A similar result was obtained for the fatty acids of the phospholipids (Fig. 2b). In the case of neutral saponifiable lipid (Fig. 2c) considerable radioactivity was incorporated into both the fatty acids of the leucocytes and red cells.

The biosynthesis of lipids in human blood is complex and it is difficult to distinguish experimentally between synthesis and exchange. From the above experiments, however, it would appear that leucocytes, although 1000 times less numerous than red cells in normal human blood, make a considerable contribution to the synthesis of lipids from acetate. They appear to be responsible for all of the synthesis of unsaponifiable lipid, and play a major part in the incorporation of acetate into phospholipids. They incorporate acetate into neutral saponifiable lipid, although in this process the red cells appear to play a considerable part. The experimental evidence suggests that in the red cell fraction the reticulocytes are playing a major part in lipid biosynthesis. It is not possible at this stage, however, to decide whether biosynthesis of lipids ceases completely with maturation of the erythrocyte; or whether there is a steady decline in activity with advancing age, *e.g.* as found by ALLISON AND BURN[7] for other red cell enzyme activities.

Since leucocytes synthesize relatively large amounts of lipid, some of which is contributed to plasma lipoprotein[3,6] it is possible that erythrocytes depend to some extent for their viability upon lipid synthesized by leucocytes. Experiments are planned to ascertain whether storage of erythrocytes *in vitro* can be prolonged by appropriate lipid supplements.



(a) (b) (c)

Fig. 2. A mixture of whole blood (100 ml) and acid–citrate–dextrose (15 ml), streptomycin-penicillin (6 mg) and sodium [1-^{14}C]acetate (50 μC) was incubated at 35°. Samples (20 ml) were withdrawn at different time intervals and treated as follows: The mixture was centrifuged and the plasma removed. The buffer layer was transferred to another tube. To the remaining cells an equal volume of a solution of sodium ethylenediamine tetra-acetate (0.01 % w/v) in saline (0.89 % w/v) was added. The buffer layer was mixed with an equal volume of 0.1 M $CaCl_2$ in saline. The cells from which the buffer layer had been removed were passed through a layer of ballotini glass beads (size 12), approximately 1″ deep. The ballotine was resting in a glass filter funnel, 3″ diameter, and held in position by a plug of glass wool in the neck of the funnel. The ballotini was then washed 3 times with 0.1 M $CaCl_2$ in saline. The combined eluate and washings were added to 20 volumes of ethanol–ether to give the "red-cell" fraction. The buffer layer was then passed through the ballotini which was then washed with a further 3 ml of $CaCl_2$ solution to remove most of the red cells. The ballotini was then added to approx. 400 ml of ethanol–ether to give the "white-cell" fraction. The lipids were isolated as described for previous experiments. ○, white cells; ●, red cells. (a) Unsaponifiable lipid; (b) Phospholipid; (c) Saponifiable lipid.

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CHROMATOGRAPHIC AND RADIOISOTOPIC INVESTIGATIONS OF THE LIPID COMPONENTS OF RUNNER BEAN LEAVES

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SUMMARY

The total lipids of runner bean leaves have been separated by chromatography on silicic acid-impregnated paper into at least eleven components. These have been partially identified by their R_F values, staining behaviour with various specific reagents, radioisotope incorporation data, and products of mild alkaline hydrolysis. Apart from pigments and glycerides, six phosphatide components were found, five of which have been identified as lecithin, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl inositol and phosphatidic acid; also, four distinct glycolipids, and a sulfur-containing lipid were detected. Hydrolysis studies showed that the four glycolipids contained galactose, glucose, glycerol and an unknown sugar in different molecular proportions, and that the sulfolipid contained glucose, the same unknown sugar, and an acid-stable form of sulfur.

INTRODUCTION

Chemical and metabolic studies of plant lipids have been greatly hindered by the lack of suitable methods for the resolution of the complex lipid mixtures encountered. Previous attempts in this laboratory[1] to separate leaf lipids by silicic acid column chromatography have not resulted in a clear-cut resolution of the components. Nevertheless, evidence derived from the analytical data and the products of hydrolysis of the fractions obtained indicated the presence of lecithin, phosphatidyl ethanolamine, phosphatidyl serine, and inositol phosphatides, and also that each fraction contained considerable amounts of lipid-bound reducing sugars (glucose, galactose, and arabinose).

Two-dimensional paper chromatography has also been used by BENSON AND MARUO[2] to separate the lipids of *Scenedesmus*. They obtained a partial resolution into four fractions, the major components of which were identified by their products of mild alkaline hydrolysis. In addition to lecithin, phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol, they discovered a new phosphatide, phosphatidyl glycerol, which was the major phosphatide component in *Scenedesmus* and was also shown to be present in higher plants. Later, BENSON *et al.*[3] reported the presence of large amounts of glycolipids and of a sulfolipid in *Chlorella*. Recently, Zill and Harmon[4] isolated two monoglycerides from spinach leaves and chloroplasts, using silicic acid column chromatography.

Chromatography on silicic acid-impregnated paper by the procedure of Marinetti *et al.*[5,6] has been used successfully to separate animal lipids, and it appeared promising as a means for resolving the complex lipid mixtures present in plants. Ferrari[7] has recently applied this technique to the [^{14}C] and [^{32}P] lipids of *Chlorella*, but did not obtain a clear-cut separation. In this laboratory, incomplete resolution was also obtained with the total lipids of runner bean leaves, using the technique of Marinetti *et al.*[6]. However, it was found that, by reducing the amount of lipid phosphorus per spot and redeveloping the chromatogram in the same solvent and in the same direction, a separation into at least eleven components was achieved. To aid in the identification of these components, chromatography was carried out with the lipids of leaves which had been labelled with ^{32}P, ^{35}S or ^{14}C (from $^{14}CO_2$, [^{14}C]glucose (uniformly labelled) or [1-^{14}C]acetate). Evidence concerning the identity of these components, based on their staining behaviour with specific reagents, radioisotopic data, and products of hydrolysis, will be presented. The results obtained here indicate that the modified chromatographic procedure should prove useful for studying lipid metabolism in plants.

MATERIALS AND METHODS

Plant material

The results reported were obtained with primary leaves of scarlet runner bean (*Phaseolus multiflorus*) grown under greenhouse conditions in soil containing vermiculite. After 14 days of growth, the tops of the plants were removed to prevent the formation of pinnate leaves.

Chromatographic standards

Samples of synthetic GPE*, GPS, and GPG were generously provided by Dr. E. Baer; GPC was a gift from Dr. C. S. MacArthur; IMP was obtained from the California Corporation for Biochemical Research; GP was synthesized by the method of Baer and Fischer[8]. G-Gal and G-Gal-Gal were a gift from Dr. H. E. Carter.

Administration of radioactive precursors

$^{14}CO_2$ was administered to individual leaves in a polyethylene bag in the light (2000 ft.c.). Sodium [1-^{14}C]acetate, [^{14}C]glucose uniformly labelled, [^{32}P]orthophosphate, or [^{35}S]sulphate were supplied to the leaf in the light (2000 ft.c.) by immersing the petiole in a solution of the labelled compound (adjusted to pH 5–6) for 30–45 min, followed by a further period in the light with the petiole immersed in tap water. Further details are given in Table I.

Extraction of total lipids

The lipids of individual "tagged" leaves were extracted with isopropanol and partitioned into chloroform as described previously[9]. The chloroform extract was,

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* Abbreviations: GP, L-α-glycerophosphate; GPC, L-α-glycerophosphoryl choline; GPE, L-α-glycerophosphoryl ethanolamine; GPS, L-α-glycerophosphoryl serine; GPI, glycerylphosphoryl inositol; GPG, glycerylphosphoryl glycerol; IMP, inositol monophosphate; G-Gal, β-D-galactopyranosyl-1-glycerol; G-Gal-Gal, α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl-1-glycerol. cycGP, cyclic-1,2-glycerophosphate; MeGP, glycerophosphate monomethyl ester.

TABLE I

INCORPORATION OF RADIOISOTOPES INTO LIPID FRACTION OF RUNNER BEAN LEAVES

Leaf No.	*Age of leaf, days*	*Precursor taken up, μC**	*Duration in the light (min)*	*Radioactivity in isopropanol extract, counts/min × 10^{-3}**	*Radioactivity in total lipids, % of isopropanol activity**	*Total lipid P, μg*	*Specific activity counts/min/μg P*
1	23	$^{14}CO_2$ (100 μC)	1	63.6	4.8	183	—
2	23	$^{14}CO_2$ (100 μC)	31	107	7.8	163	—
3	23	$^{14}CO_2$ (100 μC)	61	254	5.4	146	—
4	23	$^{14}CO_2$ (100 μC)	91	214	16.4	134	—
5	16	[^{14}C]glucose (uniformly labelled) (50 μC)	102	833	4	150	—
6	19	[^{14}C]glucose (uniformly labelled) (49 μC)	128	806	7	158	—
7	19	[1-^{14}C]acetate (36 μC)	133	303	66	103	—
8	16	[^{32}P]orthophosphate (190 μC)	110	275	49	139	970
9	19	[^{32}P]orthophosphate (169 μC)	125	110	70	206	375
10	16	[^{35}S]sulphate (180 μC)	120	142	20	127	—
11	19	[^{35}S]sulphate (82 μC)	133	96	24	160	—

* ^{32}P and ^{35}S activities are corrected for decay.

however, washed twice with 10-ml portions of a 1 *M* sodium chloride solution containing 0.25 *M* disodium phosphate plus 0.25 *M* sodium sulphate, and twice with water (15 ml). Although water-soluble radioactive substances were still present, further washing with water was avoided because of the formation of troublesome emulsions. These water soluble impurities remained near the origin on silicic acid-impregnated paper and did not interfere in the interpretation of the chromatograms. They could be virtually removed by washing the chromatogram in distilled water (see Fig. 4). The radio-activities incorporated into the lipid fractions (chloroform extracts) are given in Table I.

Chromatography of total lipids

An aliquot of the total lipid extract (washed chloroform solution) was evaporated to dryness in a stream of nitrogen, and the residue was dissolved in amyl alcohol–benzene mixture (1:1, v/v); 10 μl of this solution (containing 4–5 μg of lipid P) was applied as a spot to a 4.5″ × 18″ strip of silicic acid-impregnated Whatman 3 MM paper[5]. Chromatography was carried out at room temperature (25–26°) for 18–20 h

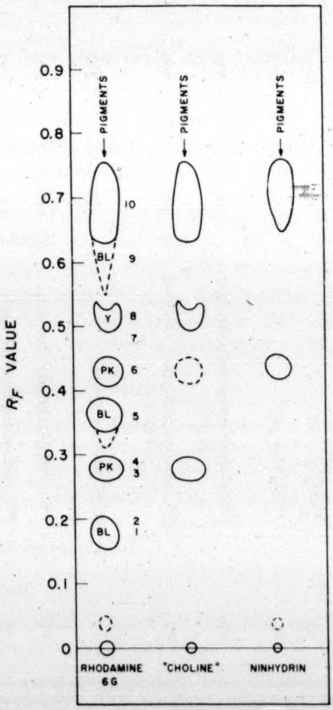

Fig. 1. Tracing of a chromatogram (single development) of total unlabelled lipids of runner bean leaves. Numbering of the spots is made to conform with that in Figs. 2 and 3. Abbreviations: Bl, blue; Y, yellow; Pk, pink; "choline" refers to the phosphomolybdate-stannous chloride stain.

in diisobutyl ketone–acetic acid–water (40:25:5, v/v), according to the procedure of MARINETTI *et al.*[6]. To obtain further resolution, the chromatogram was dried in the fume hood for 15–20 min and was immediately redeveloped in the same solvent and in the same direction for a period of 20 h. Radioactive spots were located by autoradiography of the dried chromatogram. To aid in the detection of weakly radioactive spots, the autoradiogram was scanned by means of a recording Spinco densitometer. The relative radioactivities of the spots were calculated from the areas under the peaks on the tracing obtained, as follows:

$$\% \text{ of total radioactivity in spot} = \frac{\text{area under peak}}{\text{total area of peaks}} \times 100\%$$

Total lipids were located by staining the chromatogram with Rhodamine 6G[5,6]. Amino-containing lipids were detected by spraying the chromatograms with 0.5% ninhydrin in ethanol containing 1% of a lutidine–collidine mixture (3:1, v/v).

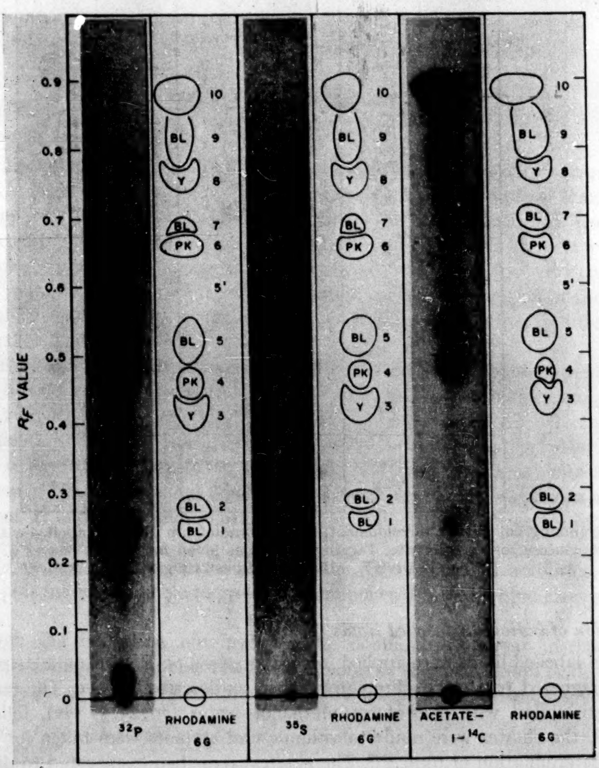

Fig. 2. Chromatogram (double development) and autoradiograph of total lipids labelled with ^{32}P, ^{35}S, or ^{14}C (from radioacetate). Tagging conditions are given in Table I (leaves 9, 11, and 7, respectively). Abbreviations as in Fig. 1.

Choline-containing lipids were detected by staining with the phosphomolybdic acid-stannous chloride reagent[10], but this reagent was found to react also with some non-choline containing lipids (see Figs. 1, 2, and 3). Lipids containing vicinal glycol groups were detected by the periodate-Schiff reagent[11].

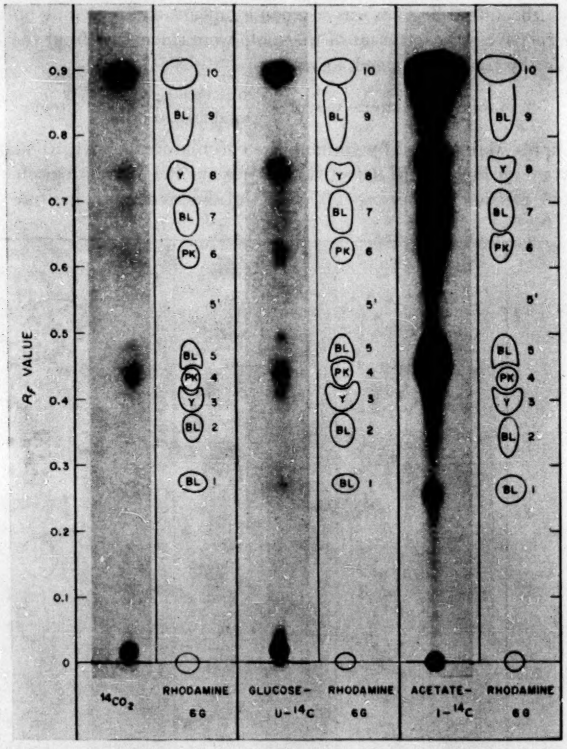

Fig. 3. Chromatogram (double development) and autoradiogram of lipids labelled with ^{14}C from $^{14}CO_2$, radioglucose, or radioacetate. Tagging conditions given in Table I (leaves 4, 6, and 7, respectively). Abbreviations as in Fig. 1.

Deacylation of individual labelled lipids

Total labelled lipids were applied in six spots (4–5 μg P/spot) on a strip of silicic acid-impregnated paper and chromatographed as described above. The radioactive areas were eluted with chloroform–methanol–water (70:25:5, v/v), followed by methanol, the eluates were made to volume, and aliquots were taken for counting and for determination of total P[12]. The eluates were then evaporated to dryness in a stream of nitrogen and the residues were dissolved in 0.1 ml of methanol–carbon tetrachloride (85:15, v/v); 0.1 ml of 0.2 *N* methanolic KOH was added and the solution was kept at 37° for 20 min. To the ice-cold solutions was added Dowex-50-H^+ (approx. 0.1 g wet wt.) and after 1–2 min the slightly acidic supernatant was removed, and the resin was washed twice with 0.1 ml of methanol. The combined supernatant and washings were neutralized with 0.1 *N* methanolic ammonium hydroxide, evaporated to a small volume, and applied to strips of HCl-washed Whatman No. 1 paper. This procedure is essentially that of DAWSON[13] as modified by BENSON AND MARUO[2,16].

The products of hydrolysis were separated by one-directional ascending chromatography in saturated phenol–water (PW; the pH was adjusted to 5.4 with sodium hydroxide) and in *n*-butanol–acetic acid–water (BAW, 5:3:1, v/v). Water-soluble phosphate esters were identified by their R_F values in these two solvent systems compared to those of authentic compounds. Other products were eluted from the chromatograms, hydrolysed with 3 *N* HCl at 100° for 1.5 h, and the hydrolyzates were chromatographed in pyridine–ethyl acetate–water (PEAW, 4:10:10, upper phase). Sugars, glycosides and polyhydric alcohols were detected with the silver nitrate–sodium hydroxide stain and identified by comparison of their R_G values (movement relative to glucose) with those of authentic compounds.

RESULTS AND DISCUSSION

Chromatographic separation of the lipids

When the total unlabelled lipids of runner bean leaves were chromatographed on silicic acid-impregnated paper by the single development technique of MARINETTI *et al.*[6], an apparent resolution into seven components was obtained (Fig. 1). However, the deacylated products of the separated spots indicated that some (*e.g.*, 1,2 and 3,4) contained more than one component, and that in general, every spot was contaminated with material from adjacent spots. Although complete resolution of the lipids was not achieved, the results obtained were nevertheless sufficiently promising to warrant further study.

When the ^{32}P, ^{35}S, or ^{14}C lipids were chromatographed by the double-development method, a further resolution was obtained, giving a total of 10 major components detectable with Rhodamine 6G, and one minor component (spot 5′) detected only on autoradiograms (Figs. 2 and 3). Comparison with the chromatogram in Fig. 1 shows that redevelopment resulted in the resolution of spot 1,2 (Fig. 1) into a phosphatide (spot 1), and a sulfolipid (spot 2); in the resolution of spot 3,4 into a phosphatide (spot 4) and a non-phosphatide (spot 3); and in the appearance of a new non-phosphatide, spot 7.

The R_F values, staining behaviour, and the ^{32}P, ^{35}S, or ^{14}C activities of the separated spots made it possible to group the components into the lipid classes given in Table II.

Although the resolution obtained by the double-development method was greater than was expected, further resolution of some of the spots would be desirable, in particular, spot 4, which still contains a mixture of a phosphatide and a glycolipid.

Identification of separated lipid components

Phosphatides: The phosphatide components were further characterized by their products of mild alkaline hydrolysis (Table III). These data, in conjunction with the R_F values and the staining behaviour of the intact phosphatide spots (Table II) led

TABLE II

CHARACTERISTICS OF CHROMATOGRAPHICALLY SEPARATED LIPID COMPONENTS

See Figs. 2 and 3. Abbreviations: w, weak; m, medium; s, strong; —, negative.

*Spot No.**	*Average R_F value*	*Rhodamine 6G stain***	*Ninhydrin stain*	*Phosphomolybdate stain****	*Periodate-Schiff stain*	*Radioisotope incorporation, % of total*§					*Lipid class*
						^{32}P	^{35}S	$^{14}CO_2$	$[^{14}C]$glucose	$[^{14}C]$acetate	
1	0.25	Blue	—	—	w	18	0	1	1	1	Phosphatide
2	0.32	Blue	—	—	m	0	100	<1	<1	<1	Sulfolipid
3	0.41	Yellow	—	s	s	0	0	<1	4	1	Glycolipid
4	0.44	Pink	—	m	s	29	0	14	9	10	Phosphatide + glycolipid
5	0.51	Blue	w	—	m	23	0	6	5	6	Phosphatide
5′	0.59	—	—	—	—	4	0	<1	3	1	Phosphatide
6	0.63	Pink	s	w	Trace	23	0	7	7	3	Phosphatide
7	0.68	Blue	—	—	m	0	0	13	12	4	Glycolipid
8	0.75	Yellow	—	s	s	0	0	14	19	12	Glycolipid
9	0.81	Blue	—	—	—	2	0	13	8	11	Phosphatide
10	0.90	Violet	—	—	—	0	0	30	33	49	Pigments + glycerides

* Most of the plant pigments were in spot 10; traces were also present in spots 7 and 9.

** Chromatograms viewed under u.v. light (366 mμ).

*** This reagent reacts with some non-choline containing lipids (MARINETTI *et al.*[6]).

§ Activity present at or near the origin of the chromatograms is due to water-soluble non-lipid material and is not included in the total activity.

to the identification of these components as given in Table III. It should be mentioned that GPI was the major phosphate ester in the hydrolysate of phosphatidyl inositol (spot 1), IMP and MeGP being just detectable on the autoradiogram. The latter two compounds probably arose by base-catalyzed transesterification and partial methanolysis, respectively, of GPI[14-16]. cycGP was found in the hydrolyzate of lecithin in amounts almost equal to that of GPC, but was completely absent in the hydrolyzates of the other phosphatides. MARUO AND BENSON[16] previously identified cycGP and MeGP in mild alkaline hydrolyzates of total ^{32}P lipids of rat liver and of various plants.

TABLE III

^{32}P PHOSPHATIDE COMPONENTS OF RUNNER BEAN LEAVES

Lipid spot No.	*% of total lipid P**	*^{32}P specific activity. relative values*	*^{32}P deacylated products*			*Probable identification of phosphatide*
			R_F values in		*Identified as***	
			PW	*BAW*		
1	9	3.0	0.14	0.02	GPI***	Phosphatidyl inositol
			(0.14)	0.07	IMP	
			0.58	0.35	MeGP***	
4	45	1.0	0.49	0.27	cycGP***	Lecithin
			0.88	0.39	GPC	
5	22	1.6	0.45	0.26	GPG	Phosphatidyl glycerol
5′	6	1.1	—	—	—	Unidentified
6	17	2.1	0.60	0.19	GPE	Phosphatidyl ethanolamine
9	1	2.8	0.35	0.33	GP	Phosphatidic acid

* Total lipid P eluted from chromatogram, 25.4 μg (blank corrected, 75 % recovery).

** R_F values for authentic phosphate esters in PW and BAW, respectively were found to be: IMP, 0.145, 0.07; GPS, 0.26, 0.12; GP, 0.34, 0.33; GPG, 0.46, 0.26; GPE, 0.60, 0.19; GPC, 0.89, 0.40.

*** R_F values reported[2,16] in phenol–water and butanol–propionic acid–water, respectively: GPI, 0.12, 0.05; MeGP, 0.54, 0.28; cycGP, 0.50, 0.20.

GP was found only in the hydrolyzate of spot 9, which is therefore presumed to be phosphatidic acid[13,16,17]. BENSON AND MARUO[2] did not detect any GP in the hydrolyzates of total lipids of several plants and concluded that phosphatidic acid was absent in them. The present results indicate that the small amount of phosphatidic acid found was actually present as such in the leaves and was not formed by the action of phosphatidase C on lecithin or phosphatidyl ethanolamine[18], inasmuch as the specific activity of the phosphatidic acid was greater than that of any of these components (Table III). Recent studies have shown that small amounts of phosphatidic acid of high specific activity are also present in [^{32}P] lipids of animal tissues[19].

The identity of spot 5′ was not ascertained because no phosphate ester could be detected on the chromatogram of the mild alkaline hydrolyzate. From its R_F value, spot 5′ appears to be phosphatidyl serine, but its negative ninhydrin reaction makes this unlikely. Phosphatidyl serine is presumed to be present in spot 5, which gave a weakly positive ninhydrin reaction. However, GPS could not be detected in the mild alkaline hydrolyzate of spot 5, presumably because of its low concentration.

The distribution of lipid P among the phosphatide components (Table III) shows that lecithin has the highest concentration, followed by phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidyl inositol; phosphatidic acid was present only in traces. Similar concentrations of the phosphatides were found in various higher plants by BENSON AND MARUO[2].

Sulfolipid and glycolipid components

Information concerning the structure of the sulfolipid and the four glycolipids was obtained by examining the products of acid hydrolysis of the corresponding deacylated products (glycosides). Deacylation of the [^{35}S]sulfolipid yielded only one ^{35}S labelled compound, and acid hydrolysis of this compound resulted in the formation of a new ^{35}S labelled compound, together with free glucose, an unknown sugar, and an unknown compound which appeared as a white spot on the chromatogram (Table IV). Also, no inorganic sulfate was detected in the acid hydrolyzate, suggesting that the sulfur is in a very stable form (*e.g.*, N-sulfate, sulfonic ester, sulfoxide, or thio ether). BENSON *et al.*[20] have concluded that the sulfolipid in *Chlorella* is a sulfonogalactosyl monoglyceride. The absence of glycerol and galactose, and the presence of glucose and two unknown compounds in the hydrolyzate indicates that the structure of the sulfolipid in runner bean leaves is more complex than that deduced by these authors. Further structural studies will depend to a large extent on obtaining sufficient quantities of the sulfolipid.

After deacylation of the [^{14}C]glycolipids (lipid spots 3, 4, 7, and 8), in each case only one ^{14}C labelled spot (glycosides a, b, c, and d, respectively) was obtained when the hydrolyzates were chromatographed either in PW or BAW (Table IV). Although glycosides a and b had R_F values similar to those of G-Gal-Gal in these solvents, and glycosides c and d had values similar to those of G-Gal, their products of acid hydrolysis (Table IV) indicate that each glycoside is either a different oligosaccharide, or else a mixture of relatively simple glycosides which are not resolved in PW or BAW*. If the first interpretation is correct, the glycolipids separated on silicic acid-impregnated paper would correspond to individual entities differing in the type of oligosaccharide moiety present in the molecule. The second interpretation would imply that each glycolipid is a mixture of two or more unresolved glycolipids containing relatively simple glycoside moieties. More detailed studies are necessary, however, before any further conclusions concerning the structure of the glycolipids can be made.

It should be noted that the present results are not in accord with the galactosyl monoglyceride structures deduced by BENSON *et al.*[3] for the glycolipids of *Chlorella*. They do agree with those of BENSON *et al.* on one point, namely that the only [^{14}C] sugar detected was galactose. However, this finding is interpreted here as resulting from the very much greater rate of turnover of galactose in the glycolipids relative to that of the other sugars. It is expected that after longer tagging periods glucose and arabinose will also become appreciably labelled, and indeed these sugars have been found labelled in a previous study of runner bean lipids[9].

Radioisotope incorporation into separated lipid components

Under the conditions employed for labelling the lipids (Table I), marked differences were found in the ^{32}P specific activities of the phosphatide components (Table

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* The latter interpretation would be in accord with the finding of a previous study[1] that three discrete glycosides were present in the deacylated products of phosphatide Fraction I (containing large amounts of glycolipids); in subsequent work these glycosides were found to consist of a glycerol arabinoside, and two galactosides (KATES, unpublished results).

TABLE IV

HYDROLYSIS PRODUCTS OF [^{35}S]SULFOLIPID AND [^{14}C]GLYCOLIPIDS

Lipid spot No.	Deacylated products	R_F value**		Products of acid hydrolysis of glycosides*					
		PW	BAW	^{35}S compound***	Glucose	Galactose	Arabinose	Unknown sugar§	Glycerol
2	[^{35}S]glycoside §§	0.19 §§ 0.23	0.04	+	++	—	—	+	—
3	[^{14}C]glycoside a	0.44	0.09	—	++	+ §§§	—	+	Trace
4	[^{14}C]glycoside b	0.45	0.09	—	+	+++§§§	—	+	+
7	[^{14}C]glycoside c	0.55	0.28	—	++	+ §§§	Trace	+	Trace
8	[^{14}C]glycoside d	0.58	0.30	—	++	+++§§§	+	+	+

* All hydrolysis products listed reduced silver nitrate, and their relative concentrations are indicated by the number of + signs; in addition, the hydrolyzate of each glycoside contained a compound (R_G in PEAW, 0.60) which did not reduce silver nitrate but appeared as a white spot on the chromatogram.

** G-Gal has R_F in PW, 0.63, and in BAW, 0.30; G-Gal-Gal has R_F in PW, 0.45, and in BAW, 0.09.

*** R_G in PEAW, 0.29. R_G of [^{35}S]sulfate, < 0.1.

§ R_G in PEAW, 1.36; as compared to xylose, 1.28, and ribose, 1.43.

§§ R_G in PEAW, 0.32. The [^{35}S]glycoside gives a double spot in PW, but each one gives identical products on acid hydrolysis.

§§§ ^{14}C labelled, both with $^{14}CO_2$ and radioglucose as precursors.

III). Phosphatidyl inositol was found to have the highest specific activity, followed in decreasing order by phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl glycerol, the unknown phosphatide (spot 5′), and lecithin. Although the present data are insufficient for any conclusions to be made concerning the biosynthetic reactions involved in the synthesis of the phosphatide components, the following points deserve attention. The fact that lecithin has only half the specific activity of phosphatidyl ethanolamine probably is partly a reflection of the greater pool size of lecithin (Table III); allowing for this, it would appear that the ^{32}P turnover rate for lecithin may actually be higher than that of phosphatidyl ethanolamine. Phosphatidyl inositol and phosphatidic acid with high specific activity has also been observed in animal tissues[13, 19] and indicates a high rate of synthesis or turnover. Recent investigations[21] have implicated phosphatidic acid as a precursor of phosphatidyl inositol, but the present results are difficult to reconcile with this conclusion since phosphatidyl

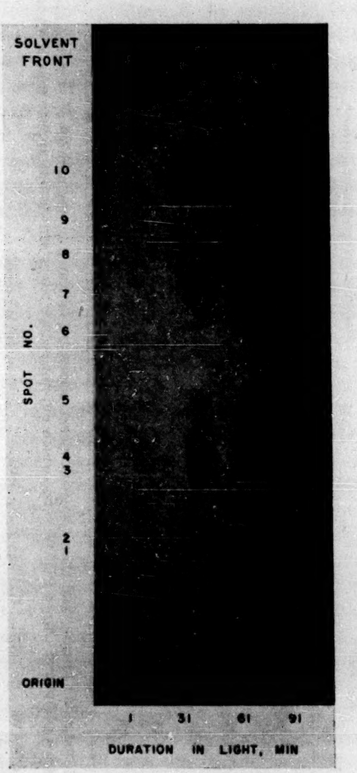

Fig. 4. Incorporation of ^{14}C into lipid components after photosynthesis in $^{14}CO_2$ for various periods. Tagging conditions given in Table I (leaves 1, 2, 3, and 4).

inositol was found to have a significantly higher specific activity than that of phosphatidic acid. It may also be concluded from the present data that the high ^{32}P specific activity in the unknown phosphatide Fraction III and in the pigment fraction found in a previous study[9] is due to the presence of phosphatidyl inositol and phosphatidic acid, respectively.

The incorporation of ^{14}C into the lipid components after photosynthesis in $^{14}CO_2$ for varying periods is shown in Fig. 4. After 1 min, no ^{14}C was detected in any of the components, but after 30 min or longer, ^{14}C was increasingly incorporated into spots 1, 4, 5, 6, 7, 8, and 10 (slight activity in spot 9 may be partly due to the presence of pigments). The proportion of activity in these spots (see Table II) appeared to be fairly constant during the period studied (30–90 min). Apart from the pigment spot, incorporation into glycolipids b and d (spots 4 and 8) was quite pronounced. This may be correlated with their high content of galactose (Table IV). Similarly, the low activity in the sulfolipid and in glycolipids a and c (spots 2, 3 and 7) may be correlated with the low galactose contents of these glycolipids and the absence of galactose in the sulfolipid. The ^{14}C activity in spots 5 and 6 was partly associated with the corresponding deacylated products (GPG and GPE, respectively). No ^{14}C labelled deacylated products of the other phosphatides could be detected.

The ^{14}C incorporation pattern with radioglucose as precursor was remarkably similar to that obtained with $^{14}CO_2$ as precursor (Table II). This would suggest that in both cases ^{14}C incorporation into the galactose moiety of glycolipids is preceded by a rapid conversion of glucose ⟶ uridine diphosphate glucose ⟶ uridine diphosphate galactose, as has been deduced previously by BENSON *et al.*[3]. The primary precursor of galacto-lipids in animal tissues has recently been shown to be uridine diphosphate galactose[22], but in this case the lipids synthesized were cerebrosides.

^{14}C from radioacetate was incorporated into each of the components in proportions similar to those obtained with $^{14}CO_2$ or radioglucose. It may be assumed from the results of a previous study[9] that the ^{14}C from radioacetate was incorporated exclusively into the fatty acid moieties of the lipids. It is therefore surprising that so little activity was associated with the sulfolipid and glycolipid a. Since the intensity of the Rhodamine 6G stain shows these compounds to be present in high concentration, their low ^{14}C activities suggest that they contain fewer fatty acids per molecule than the other lipids.

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PLANT PHOSPHOLIPIDS

III. IDENTIFICATION OF DIPHOSPHATIDYL GLYCEROL

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SUMMARY

A linear polyglycerophosphate has been identified among the deacylated phosphatides from *Chlorella*, *Rhodospirillum rubrum* and higher plants. It was identified as 1,3-diglycerophosphorylglycerol by comparison of its chemical and physical properties with those of the synthetic ^{32}P labeled compound. The original plant lipid appears to be identical with cardiolipin from animal tissues, indicating that both are diphosphatidyl glycerols.

INTRODUCTION

Phosphatidyl glycerol is a major phospholipid of plants[1] and is particularly concentrated in chloroplast and bacterial chromatophore structures[2]. The active transphosphatidylation observed during photosynthesis[3] suggested the possible occurrence of other polyglycerol phosphatides in plants. In this communication we report the

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The following abbreviations will be used in this article: GP, glycerophosphate (D-glycerol-1-phosphate); GPI, glycerophosphoryl inositol; GPG, glycerophosphoryl glycerol; GPC, glycerophosphoryl choline; GPS, glycerophosphoryl serine; GPE, glycerophosphoryl ethanolamine; GPGPG, 1,3-diglycerophosphoryl-glycerol; PGP, glycerol-1,3-diphosphate, PGPG, 3-glycerophosphoryl glycerol-1-phosphate; PW, phenol–water; BPAW, butanol–propionic acid–water.

identification of diphosphatidyl glycerol in *Chlorella*, *Scenedesmus*, *Rhodospirillum rubrum* and higher plants.

MATERIALS AND METHODS

Preparation of radiophosphatides from Chlorella *and* Scenedesmus

^{32}P labeled lipids from *Scenedesmus* and *Chlorella* were prepared as described previously[1]. [^{14}C]phosphatides were similarly isolated from *Chlorella* which had photosynthesized for 6 h in $^{14}CO_2$.

Deacylation of phosphatides

Phosphatides were deacylated in 0.1 *N* methanolic potassium hydroxide at 37° for 15 min[1,4]. After neutralization with Dowex 50-H$^+$ resin, the phosphate diesters were separated by chromatography on Whatman No. 4 paper in phenol–water (100:38), PW, and in butanol–propionic acid–water (142:71:100), BPAW.

Detection and identification of phosphate esters

The methanolysates from [^{32}P]*Chlorella* and *Scenedesmus* revealed GPGPG[5], in addition to the previously identified phosphate diesters[1]. The radiogram in Fig. 1 shows that GPGPG has R_F values of 0.14 in PW and 0.07 in BPAW, nearly identical with those of GPI. [^{14}C]GPGPG was obtained similarly from [^{14}C]*Chlorella*. Corn and clover phosphatide methanolysates were chromatographed and revealed on paper by phosphate spray reagent or periodate-Schiff's spray reagent[6]. [^{32}P]GPGPG isolated from *Chlorella* cochromatographed precisely with the corresponding spot on sprayed chromatograms of deacylated corn phosphatides.

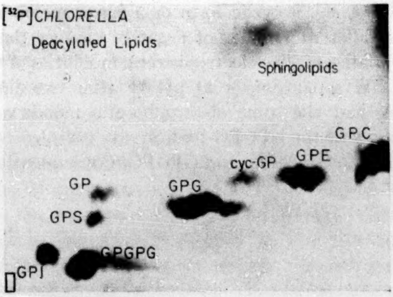

Fig. 1. Radiogram of deacylated phospholipids of [^{32}P]*Chlorella*. High concentration of GPGPG is the result of culture in P-deficient medium followed by restoration of the normal phosphate level. Chromatogram was developed in x-direction with phenol–water and in y-direction with butanol–propionic acid–water.

Chromatograms of methanolysates of lipids extracted from *R. rubrum* chromatophores were neutron-activated[7] to reveal phosphorus-containing compounds. Products were identified by addition of [^{32}P]*Chlorella* lipids to the chromatophore lipids prior to deacylation. The glycerophosphoryl esters were located by virtue of their ^{32}P activity. This was then allowed to decay completely. The papers were then treated with $3 \cdot 10^{16}$ neutrons/cm^2 and re-radioautographed. The products revealed were GPG, GPE and GPGPG with a smaller amount of GPI and a negligible amount of GPC. The phosphorus content was estimated by comparison with the activity induced in 1.0 μg ^{31}P standards placed on the chromatograms. The concentration of GPGPG lipid in several preparations of wet packed chromatophores was 0.8 to $1.5 \cdot 10^{-3}$ M compared to $6 \cdot 10^{-3}$ M GPG lipid and 1.5 to $9 \cdot 10^{-3}$ M GPE lipid (cephalin).

Acid hydrolysis of GPGPG

[^{32}P]GPGPG from [^{32}P]*Chlorella* was hydrolyzed in 0.1 N hydrochloric acid at 100° for 5, 15, and 30 min. Hydrolysates were evaporated on Whatman No. 4 paper without neutralizing the excess acid and chromatographed in PW and in BPAW. The radiogram of the 5-min hydrolysate revealed five spots. Compound III (R_F 40, 17)[*] was identified as GPG. Compound II (R_F = 32, 22) was identified as α-GP by cochromatography; compound I (R_F 33, 28) was probably β-GP. Spot V (R_F = 14, 7) was unhydrolyzed GPGPG. A number of data indicated that spot IV (R_F = 30, 7), which had the same R_F as spot V in BPAW and twice the R_F of spot V in PW, was also GPGPG. The chloride ions left on the paper after loading the HCl hydrolysate were shown to have caused the GPGPG to form a double spot. Chromatography of GPGPG with added sodium chloride gave the same double spot. Furthermore, when spot IV was eluted from the chromatogram and rechromatographed, the compound showed the R_F's typical of GPGPG. Any likely intermediate in the hydrolysis of a homologue of GPGPG, *e.g.* a cyclic form or a lower member of the homologous series, should have a different number of negative charges than the unhydrolyzed material at an appropriate pH. The electrophoretic mobilities of GPGPG and spot IV at pH 7.7 after the PW separation or at pH 12 after two-dimensional separation showed that spot IV had the same electrophoretic mobility as GPGPG. Direct electrophoresis at pH 7.7 of the GPGPG hydrolysate revealed only compounds with mobilities corresponding to GP, GPG, and GPGPG. Consequently, spot IV must have been GPGPG.

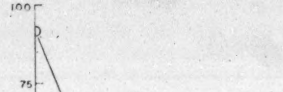

Fig. 2. Hydrolysis of GPGPG in 0.1 N hydrochloric acid at 100°. ○, GPGPG; △, GPG; ◒, α-GP; ●, β-GP.

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[*] R_F values are given in per cent for PW and BPAW respectively.

GPGPG hydrolyzed rapidly in 0.1 *N* hydrochloric acid at 100° (Fig. 2). In fact, simply applying GPGPG to the origin in 0.1 *N* hydrochloric acid resulted in 10% hydrolysis. The zero time hydrolysate gave equal yields of both α-GP and GPG. At longer times the α-GP continued to increase while GPG gradually decreased. At 5-min hydrolysis and later some β-GP was also detected. Hydrolysis of the cyclic intermediate would be expected to produce both esters. The observed kinetic behavior would be expected in the hydrolysis of GPGPG. GP would accumulate because of its resistance to hydrolysis. GPG would be expected to accumulate to a small extent at short times and gradually disappear since experiments showed that GPG was hydrolyzed nearly as rapidly as was GPGPG.

Hydrolysis of ^{14}C labeled material in 1.0 *N* hydrochloric acid for 20 min at 100° confirmed the above reaction. The chromatographed hydrolysate yielded 1.9 times as much [^{14}C]GP as [^{14}C]glycerol.

TABLE I

PAPER ELECTROPHORETIC MOBILITY OF DIGLYCEROPHOSPHORYL GLYCEROL

Mobility of GPGPG relative to	*pH 2.0*	*pH 7.7*	*pH 12.0*
GP	1.6	0.6	0.7
GPG	1.5	1.6	1.6

Paper electrophoresis of GPGPG

Additional information on the phosphate ester linkages in GPGPG was obtained by comparison of the electrophoretic mobility of GPGPG with those of α-GP and GPG at pH 2.0, pH 7.7 and pH 12.0 (Table I). At pH 2.0 both α-GP and GPG carried an average charge of approximately —1. Since electroendosmosis was a minor effect at low pH's, molecules of approximately the same size should have about the same mobility if they have the same charge. At pH 2.0 GPGPG moved 1.6 times as fast as α-GP and 1.5 times as fast as GPG, suggesting that GPGPG has a —2 charge. At higher pH values the evaluation of the charge was less certain because of the more pronounced influence of size on mobility due to the greater electroendosmosis. At pH 7.7 GPGPG moved 0.6 times as fast as α-GP, which was doubly charged, and 1.6 times as fast as the singly charged GPG. GPGPG appeared to be doubly charged at pH 7.7 and to have a larger size than α-GP and GPG. Similar results were obtained at pH 12.0. GPGPG appeared to possess a —2 charge at pH values sufficiently high to allow ionization of the primary hydrogens from phosphoric acid esters. The GPGPG has two phosphate diester groups, which is consistent with the structure determined by acid hydrolysis.

Synthesis of [^{32}P]GPGPG

Glycerylation of [^{32}P]PGP by glycidol produced [^{32}P]GPGPG. [^{32}P]PGP was formed by [^{32}P]phosphorolysis of a mixture of 1,3-dichloro-2-propanol (5 μl) and trisodium phosphate (14 mg) in the presence of 1 mC [^{32}P]phosphate at 27° for 30 h.

The reaction products were separated by two-dimensional paper chromatography. The diphosphate area (chromatographic position of hexose diphosphates) was eluted and rechromatographed. Of the products, only one had both the chromatographic and electrophoretic properties anticipated for PGP ($R_F = 0.06$ in PW; $R_F = 0.13$ in BPAW). It was resistant to acid hydrolysis. 10 min in 1 *N* hydrochloric acid at 100° readily hydrolyze α-hydroxylated phosphate diesters while only 0.3 % of the PGP hydrolyzed to yield approximately equal activities in P_i and GP.

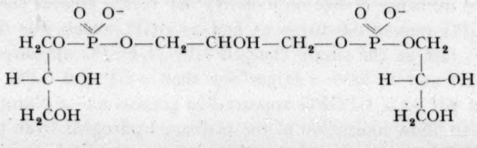
The [^{32}P]PGP solution was evaporated to dryness and treated with excess glycidol for three days at room temperature. A radiogram revealed products corresponding to residual PGP, PGPG, and GPGPG. Glycerylation of PGPG formed GPGPG. The synthetic [^{32}P]GPGPG cochromatographed precisely with GPGPG from hydrolyzed corn phosphatides and co-electrochromatographed with GPGPG from [^{32}P]*Chlorella*. The hydrolysate of synthetic [^{32}P]GPGPG after 5 min at 100° in 0.1 *N* hydrochloric acid gave the same products as did that from [^{32}P]*Chlorella*.

Silicic acid paper chromatography of [^{32}P]phosphatides

Radiophosphatides were separated by the method of MARINETTI *et al.*[8] on silicic acid-impregnated Whatman No. 1 paper in the diisobutyl ketone–acetic acid–water solvent system. GPGPG lipid was identified as one of the fast moving lipids ($R_F = 0.8$) by elution and deacylation to give GPGPG upon two-dimensional chromatography. The high R_F of GPGPG lipid indicates that it has more than two fatty acid residues. Even though GPI and GPGPG have almost the same chromatographic properties, GPI lipid, which has only two fatty acid residues, is a slow moving lipid ($R_F = 0.2$). GPGPG lipid probably would need four or five fatty acid residues to give it its high R_F.

RESULTS AND DISCUSSION

Methanolysates of radiophosphatides from [^{32}P]*Chlorella* revealed, in addition to GPI, GPC and GPG, an unknown phosphorus-containing compound not previously reported in plants. This compound, which was identified as GPGPG, is the product of deacylation of diphosphatidyl glycerol. This lipid was found in varying concentrations in all plants investigated, including *Scenedesmus*, *Rhodospirillum rubrum*, clover and corn. The structure of the deacylated lipid has been established as:



$$\begin{array}{ccccc}
 & \mathrm{O\ \ O^-} & & \mathrm{O\ \ O^-} & \\
 & \diagdown\!\diagup & & \diagdown\!\diagup & \\
\mathrm{H_2CO-} & \mathrm{P} & \mathrm{-O-CH_2-CHOH-CH_2-O-} & \mathrm{P} & \mathrm{-OCH_2}\\
| & & & & | \\
\mathrm{H-C-OH} & & & & \mathrm{H-C-OH}\\
| & & & & |\\
\mathrm{H_2COH} & & & & \mathrm{H_2COH}
\end{array}$$

Assignment of this structure is based on a series of radiochemical observations. Base-catalyzed methanolysis of [^{32}P] plant phosphatides gave a chromatographically separable product which yielded GPG and GP upon brief acid hydrolysis and predominately GP at longer times. Hydrolysis of [^{14}C]GPGPG yielded two moles of GP for each mole of glycerol. The kinetics of acid hydrolysis of GPGPG, Fig. 2, are consistent with the following sequence:

$$\mathrm{GPGPG} \xrightarrow{\mathrm{H^+}} \mathrm{GPG + GP}$$
$$\mathrm{GPG} \xrightarrow{\mathrm{H^+}} \mathrm{GP + Glycerol}$$

Experiments showed that GPG was hydrolyzed nearly as rapidly as GPGPG. Electrophoresis data suggest that GPGPG contains two diesters of phosphoric acid.

The structural assignment was confirmed by the synthesis of [^{32}P]GPGPG and cochromatography with that separated from corn phosphatides. [^{32}P]GPGPG was prepared by reaction of [^{32}P]PGP with excess glycidol. PGP was isolated from the reaction products of 1,3-dichloro-2-propanol and excess [^{32}P]trisodium-phosphate.

The number of fatty acid residues in GPGPG lipid has not been determined unequivocally. However, the high R_F in diisobutyl ketone–acetic acid–water solvent system suggests four or five fatty acid residues per lipid molecule. GPGPG lipid may be tentatively assigned the structure of a diphosphatidyl glycerol. GPGPG lipid from plants may be identical with cardiolipin obtained from animals. MACFARLANE[5] has shown that the skeleton of cardiolipin[9] is probably GPGPG and that the lipid contains four fatty acid residues. Furthermore, GPGPG lipid is found in high concentration in beef heart, sheep heart and pig heart mitochondria[10, 11].

In *Chlorella* and *Scenedesmus* GPGPG lipid normally constitutes only a small percentage of the total lipids. However, the concentration of this lipid is dependent on the metabolic state of the algae. When *Chlorella* are grown in phosphate deficient medium for several generations, the GPGPG lipid concentration increases dramatically[12]. The chromatophores of the photosynthetic bacterium *Rhodospirillum rubrum* contain high concentrations of GPGPG lipid. It seems possible that diphosphatidyl glycerol may be intimately concerned with the function of photosynthetic structures. In addition to an orienting action based on the unique di-ionic character of the lipid[10], it may serve like phosphatidyl glycerol as a "solid state metabolite" in the lamellae.

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THE ISOLATION OF CYTOCHROME *c* BY SALT EXTRACTION

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SUMMARY

A method for the isolation and purification of cytochrome *c* is presented. In this procedure a neutral salt solution is employed for the extraction of the cytochrome *c*. The extracted material is then purified with the use of ion exchange-resins.

INTRODUCTION

Recent observations have indicated that the methods of isolation of cytochrome *c* which employ strong acid[1,2] at least partially degrade the compound [3-6]. These observations led to the belief that a method for isolation and purification of cytochrome *c* which did not employ strong acids or bases might yield a superior product. Such a procedure was developed and was previously reported[7]. It is the purpose of the present paper to outline in detail the procedure employed.

Extraction of cytochrome c

Beef-heart muscle is stripped of connective and adipose tissue. The tissue is run twice through a meat grinder and the ground tissue weighed to the nearest gram. It is then extracted, using 1500 ml of a solution of 20% sucrose containing 0.25% saponin per kg heart muscle. This extraction is carried out with stirring for 30 min.

The extracted residue is separated from the extract by squeezing through No. 90 cheesecloth and discarding the extract. The residue is re-extracted once more, using 1200 ml of the sucrose–saponin solution for each kg of the original heart muscle, again discarding the extract.

The residue is then treated with 1500 ml acetone/kg of starting heart-muscle brei and is separated from the extract in the same manner as described above. The extraction is repeated using 1200 ml acetone/kg tissue. Each extraction is made within a 15 min period.

The residue from the acetone extraction is then treated with 1 l 0.1 *M* NaCl/kg of starting tissue. The tissue is extracted with this solution for 45 min with stirring and the extract separated from the insoluble residue by again squeezing through cheese-cloth. This extract contains the crude cytochrome *c*.

The tissue is re-extracted with the same volume of 0.1 *M* NaCl and the extracts are combined. The combined volume is measured and then adjusted to pH 4.5, using glacial acetic acid. A flocculent precipitate is formed. For each liter of the acidified extract, 250 ml acetone are cautiously added in a thin stream with constant stirring. After a standing period of at least 60 min at 20 to 25°, the mixture is filtered or centrifuged. A clear orange-yellow filtrate of crude cytochrome *c* results.

Purification

The crude cytochrome *c* is adsorbed on the ammonium form of the ion-exchange resin XE64, which has been previously buffered to pH 6.0. This adsorption step can be accomplished by either a column or batch procedure.

For the column procedure, a column of the resin 10 cm in height and 5 cm in diameter is prepared in a glass tube. The extracts from 5–8 kg of heart muscle are allowed to pass through the resin bed at a rate of 500–1000 ml/h. The cytochrome *c* forms a solid red band near the top of the resin. After the entire crude extract has passed through the column, the resin can be washed with 2.5 l of distilled water and at a rapid rate until the eluate is protein-free.

The batch procedure was found more convenient. To each liter of crude cytochrome *c* 7.5 g of resin are added and the mixture stirred for 45 min. After the cytochrome *c* has been adsorbed on the resin, the resin is poured into a large beaker. The resin is then washed free of extraneous protein by decantation with distilled water. The resin is then poured into a column 3.5 cm in diameter, using distilled water to wash the resin into the glass tube.

The cytochrome *c* is then eluted from the resin, using 0.5 M Na_2HPO_4. A flow rate of less than 50 ml/h assures maximum concentration of the cytochrome *c* in the eluate. The retention volume is 300 ml. The concentrated cytochrome *c* can be collected in about 100 ml of the eluate.

The cytochrome *c* is then dialyzed for 12 h at 2° against two changes of 20 vol. distilled water. Any resulting precipitate is removed by centrifugation. The cytochrome *c* is then further purified by chromatography on the sodium form of the resin at pH 6.0. A column 1 cm × 20 cm is washed free of buffer with distilled water and the cytochrome *c*, adjusted to pH 6.0. is allowed to enter the resin bed. After all the cytochrome has entered the column, a solution of 0.1 M Na_2HPO_4 is used to elute the cytochrome *c*. The major portion of the purified cytochrome *c* is eluted after 200 ml of the Na_2HPO_4 solution has entered the column. The cytochrome *c* is collected in a volume of about 150 ml. The last chromatographic step can be repeated to obtain a highly purified cytochrome preparation.

The cytochrome *c* obtained by this salt extraction procedure resulted in reasonably good yields of cytochrome *c*. In ten different preparations, the yield of purified cytochrome *c* from beef heart muscle averaged $6.5 \cdot 10^{-6}$ moles/kg. When the same procedure was applied to kidney, the yields were about $1 \cdot 10^{-6}$ moles/kg.

Properties

The cytochromes *c* isolated by different procedures were compared by electrophoresis, employing a previously published procedure[8]. As shown in Table I, the cytochrome *c* prepared by an acid-extraction procedure has a greater electrophoretic mobility than a purified salt-extracted preparation.

The highly purified cytochrome *c* obtained was assessed for its spectral properties. Table II lists the results obtained with the compound in both the oxidized and reduced form. Although the ratios selected cannot in themselves be considered as an index of

TABLE I

A phosphate buffer, pH 6.8 and ionic strength 0.05 was used and a field strength of approximately 10 V/cm was imposed.

Preparation	*Apparent mobility cm/h*
KEILIN–HARTREE preparation	1.8
Purified fraction*	1.8
Salt extracted	1.5

* A purified fraction obtained from KEILIN-HARTREE preparation by the method of MARGOLIASH[3].

TABLE II

SPECTRAL PROPERTIES OF SALT-EXTRACTED CYTOCHROME *c* PREPARATION

See text for definitions of the stages of purification of the salt-extracted cytochrome *c*.

	550 red. / 550 ox.*	*550 red. / 280 ox.*	*415 red. / 550 red.*	*415 ox. / 407 ox.*	*407 red. / 550 red.*	*415 red. / 280 ox.*
Stages of preparation:						
Salt-extracted cytochrome *c*						
Crude	1.47	0.01	3.14	1.06	2.97	0.03
Concentrated	2.56	0.60	5.41	1.25	4.32	3.25
Purified	2.86	0.91	5.33	1.32	4.03	5.03
Highly purified	3.31	1.25	4.96	1.34	3.70	6.19
KEILIN-HARTREE cytochrome *c***	3.0	1.0				5.1

* Designations such as 550 red./280 ox. refer to absorbancy ratios at the corresponding wavelengths in mμ; "red." and "ox". refer to the state of the cytochrome *c* sample at the wavelength indicated.

** Based on the average data from four preparations made in this laboratory by the method of KEILIN AND HARTREE.[2]

purity, they do help to evaluate the presence of contaminating hemoproteins and other extraneous protein. The spectral properties compare favorably with those reported by others for highly purified preparations of cytochrome *c* (see ref. 9–11).

The spectral properties of the purified preparation were also investigated in the visible region at the temperature of liquid air. The procedure was similar to that reported by ESTABROOK[12]. At this temperature, the spectrum is sharpened and intensified and the spectrum of the salt-extracted preparation is comparable to that obtained with the acid preparation of cytochrome *c* (see ref. 12). The α absorption peak is resolved into 549 and 546 mμ maxima, and the absorption in the β region has maxima at 525, 519 and 508 mμ.

The prosthetic group of the salt-extracted cytochrome *c* was also investigated. The hematins were cleaved from the protein and chromatographed as previously described[13]. Two hematins were found to be present. These results are similar to those obtained with cytochrome *c* purified by the MARGOLIASH procedure[14].

DISCUSSION

The sucrose-saponin washes are employed to disrupt the cells, particularly the erythrocytes present, while keeping the mitochondria intact. This makes it possible to wash out the bulk of the soluble cytoplasmic proteins, while the cytochrome *c* remains in the particulate residue. The acetone disrupts the particulate, leaving the cytochrome *c* unextracted in the residue. The cytochrome *c* can then be extracted with the dilute NaCl. The acidification to pH 4.5, followed by the addition of acetone, removes a great deal of extraneous protein, particularly hemoglobin and myoglobin. In the method of HAGIHARA *et al.*[14] the pH is lowered to 4.5 prior to the extraction of cytochrome *c*.

The acidification and addition of acetone also serve a second function. At pH values below 6, the elution volume for cytochrome *c* is very high[15]. Thus, the low pH increases the retention of the cytochrome *c* by the resin. Solvents, such as acetone, decrease the ionic strength of the salt solution. Consequently, the low pH and the presence of acetone both make possible the adsorption of the cytochrome *c* and its retention on the ion-exchange resin, despite the large volumes employed and the presence of relatively high concentrations of salts.

The extraction of cytochrome *c* from the heart muscle residue, after the treatment with sucrose-saponin and acetone, can be achieved with any one of a variety of salts (ammonium acetate, Na_2SO_4, NaCl). However, little additional cytochrome is extracted when the salt concentration is raised above 0.1 *M*. TSOU[16], using a heart-muscle brei, has reported similar findings.

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Short Communications

Fractionation of ribonucleic acids by countercurrent distribution

The complexity of RNA preparations from mammalian tissues has been adequately demonstrated by BRADLEY AND RICH[1] using anion-exchange chromatography. Little information was available about the nature of the products separated although MIURA AND SUZUKI[2] claimed to have separated yeast RNA into fractions of different molecular weight but of the same base composition by ion-exchange chromatography on Dowex-2.

Rat- and chicken-liver RNAs can be fractionated by addition of 2-butoxyethanol to solutions in 2 % potassium acetate[3,4]. Three fractions were obtained which differed in base composition and in the curves produced by counter-current distribution. This method, used analytically, demonstrated that the fraction with the highest proportion of guanine tended to remain in the aqueous phase, while the fraction with the greatest proportion of adenine travelled in the organic phase. It was also demonstrated that these fractions inter-acted in solution.

While the analytical approach showed there were a number of different RNAs in any one sample it seemed essential to recover the RNA after counter-current distribution and examine the base compositions of the fractions. Dialysis of the contents of the contents of the tubes resulted in a disappointingly low recovery and a new method which concentrates the RNA in a small volume, from which it is easily recovered, has been developed.

Fraction 3 from rat- and chicken-liver RNAs (prepared by the phenol–water method[5]) and yeast RNA have been examined by this method. The solvent system consisted of phosphate buffer, pH 7.5 (approx. 2.5 *M*) (1 vol.), water (1 vol.) and organic solvent (0.8 vol.). The organic solvent was a mixture of 2-ethoxyethanol, 2-butoxyethanol and NN-dibutylaminoethanol (100:50:7.5). Addition of this amine was essential to obtain good partitions and fractionations.

500 mg RNA were distributed in this solvent system using a Quickfit and Quartz fully automatic counter-current distribution machine (10 ml each phase) and performing 50 transfers. The contents of each 5 tubes were pooled, producing 10 fractions. The RNA was recovered as follows: the 2-phase system was extracted with peroxide-free ether, when all the RNA was transferred to the aqueous layer. This was separated off and mixed with 0.5 vol. 2-methoxyethanol when the RNA was transferred to the organic layer. The aqueous phase, containing most of the phosphate, was run off. Addition of an equal volume of *n*-butanol to the organic layer resulted in the RNA being concentrated in an aqueous layer of 10–12 ml which was then dialysed and the RNA recovered by addition of ethanol and potassium acetate. After drying and weighing about 60 % of the original material was recovered in this manner. Almost nothing was present in fraction 10 of the 3 different RNA's.

Abbreviation: RNA, ribonucleic acid.

The base compositions of some of the fractions are shown in Table I. It will be noted that in all cases the guanine is present in greatest amount in fraction 1, while adenine predominates in fraction 9. Cytosine decreases with guanine in the rat and chicken liver RNA's while, rather remarkably uracil shows very little change in the fractionation. The purine content of the fractions varies from about 52 % to about 63 %.

The basis of the separation by counter-current distribution and solvent fractionation becomes somewhat clearer, for it is quite obvious that polynucleotides with a high adenine content are much more soluble in this type of organic solvent than those with a high guanine content.

It was necessary to demonstrate that this fractionation was genuine and not a result of fragmentation during the counter-current distribution. The curves obtained by distributing the original material (Fig. 1) and fractions 1, 5 and 9 (Fig. 2) in the same solvent are evidence that no degradation has taken place during the original fractionation.

TABLE I

Source	*Fraction*	*Guanine*	*Adenine*	*Cytosine*	*Uracil*
Rat-liver RNA	1	40.0	12.6	35.1	13.3
Fraction 3	2	36.1	15.8	32.4	15.7
	5	34.2	21.6	27.0	17.2
	9	27.7	35.5	21.6	15.2
Chicken liver RNA	1	40.6	11.9	34.8	12.7
Fraction 3	2	41.0	12.0	32.0	15.0
	5	36.4	18.7	29.0	15.9
	9	29.6	33.7	21.2	15.5
Yeast RNA	1	38.6	16.7	22.6	22.1
	2	34.1	19.4	23.0	24.3
	5	25.7	28.0	22.0	24.3
	9	19.7	42.1	17.9	20.2

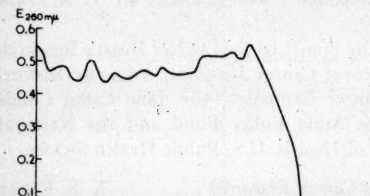

Fig. 1. Counter-current distribution curve of rat-liver RNA, fraction 3; 80 transfers.

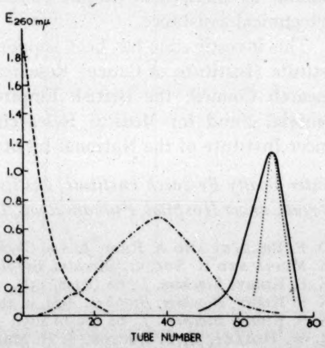

Fig. 2. Counter-current distribution curves of the fractions 1, 5 and 9 from rat-liver RNA, fraction 3; 80 transfers in each case. - - -, fraction 1; —·—, fraction 5; ——, fraction 9; . . ., theoretical curve for a single compound of the same partition coefficient as fraction 9.

As found previously[4] the height of the peak in tube 0 of fraction 1 was greater than might have been expected from an examination of the original curve, demonstrating the presence of complexes in the original material. The most remarkable feature was that the curve for fraction 9 nearly coincided with the calculated curve for a pure component. Moreover, fraction 9 from chicken appeared to be identical with fraction 9 from rat liver on the basis of the distribution curves, differences in base compositions being accounted for by a higher content of other components in the chicken-liver fraction.

It must be noted that rat liver RNA as isolated by the phenol–water method is somewhat degraded and this degradation can be lessened by incorporation of 0.5 % naphthalene-1,5-disulphonate in the homogenising medium[4]. In this case a lower proportion of fraction 3 was obtained by 2-butoxyethanol fractionation. It is possible, therefore, that fraction 9 obtained by the present method is a segment of an RNA that was originally a larger molecule.

This, however, may be an advantage, for though it has been isolated in only small amounts (about 1–2 % of the fraction 3) it may be possible to attempt some base sequence analyses. This should be easier with a smaller than with a larger RNA and could ultimately be related to the larger RNA.

The results demonstrate the usefulness of a preliminary solvent fractionation followed by counter-current distribution and particularly of the selectivity of the latter method considering the relatively small number of transfers that have been carried out. Moreover, since the distribution method separated RNA's of different base composition, while fractionation by ion exchange depends more upon differences in molecular weight, a combination of both methods may lead to the separation of a number of single compounds from RNA.

These fractionations and recoveries may be relevant in the field of protein synthesis, where HOLLEY, DOCTOR, MERRILL AND SAAD[6] have already shown that the RNA incorporating alanine into proteins can be separated by counter-current distribution from the RNA incorporating tyrosine.

The counter-current distribution machine was purchased through funds made available by the British Empire Cancer Campaign. I wish to thank Mr. E. A. COOK for technical assistance.

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Nucleotides of *Hevea brasiliensis* latex

The pyrophosphate components

The presence in latex of purine and pyrimidine material of low molecular weight was observed during the isolation of *Hevea* ribonucleoprotein[1]. Some of it was shown to be typical of hydrolysis products of nucleic acid but paper-electrophoresis patterns brought to light the occurrence, in much smaller amounts, of components with an appreciably higher negative charge. The possibility of these being of pyrophosphate character is of interest because of the recent demonstration that a polyisoprenoid biosynthetic mechanism is present in fresh latex[2] and of the implied requirement for a source of energy in the system.

In the present investigation fresh latex serum was obtained from Malaya, as previously described[1], in the form of centrifuged-serum solids (freeze dried). The presence of di- and triphosphate nucleotide fractions in the $HClO_4$-soluble fraction of this material was readily demonstrated by incubating an aliquot with hexokinase, glucose and a little $MgCl_2$ at 37° for 30 min. This and an untreated aliquot were separately adsorbed onto Dowex-1 anion-exchange resin at pH 7.5 and the mono-, di- and triphosphate derivatives progressively eluted by increasing the acidity and the ionic strength[3]. Fig. 1 shows the result of a typical experiment in which an increase in the nucleotide diphosphate fraction takes place in the hexokinase-treated specimen at the expense of an approximately equivalent amount of triphosphate. This is strongly indicative of the presence of ADP and ATP. The fact that only a portion of the triphosphate content is so converted suggests the presence of other triphosphate nucleotides. This assumption receives confirmation from the high value of the extinction maximum in the first 10% of the volume of each fraction eluted, indicating a preponderance of cytidine di- and triphosphates (which are eluted much faster than those of adenosine) in these initial collections.

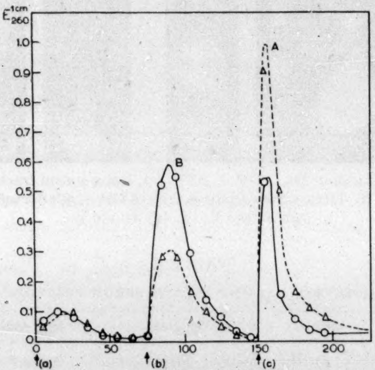

Fig. 1. Curve A, untreated extract; Curve B, hexokinase-treated extract. Elutions: at (a) with 0.003 *M* HCl; at (b) with 0.01 *M* HCl + 0.02 *M* NaCl; at (c) with 0.01 *M* HCl + 0.2 *M* NaCl.

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CDP, cytidine diphosphate; CTP, cytidine triphosphate; UDP, uridine diphosphate.

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A purified pyrophosphate nucleotide fraction was therefore prepared from latex-serum preparation by extraction at 5° with 1 *N* HCl followed by precipitation of sulphydryl components[4] with $HgCl_2$, removal of mercury with H_2S, removal of excess H_2S and final precipitation with barium acetate at pH 8.0. The barium phosphates were treated with $HClO_4$ and nucleotide derivatives eluted onto a charcoal–Celite column, washed free from inorganic phosphates, then eluted with 5 % aq. pyridine[5], concentrated *in vacuo* and applied to No. 1 filter paper for chromatography with Pabst solvent III (see ref. 6) or for electrophoresis at pH 3.2 (see ref. 7). Application of the Dische test to this concentrate gives a negative reaction, indicating the absence of deoxy-pentose derivatives.

The u.v. absorption photograph in Fig. 2 illustrates the electrophoretic results. Comparison of the three major spots obtained from the latex-serum extracts with the various markers indicates that substance 1 is largely ADP, substance 2 largely CTP and substance 3 mainly UDP. Confirmation of these conclusions follows from examination of the R_F values and from the spectral data of eluates of the various spots, given in Table I.

The chromatograms show that in addition to substances (in spot A, Table I), mainly concentrated in the position corresponding to ADP marker (R_F, 0.37), a major spot (C) occurs in the cytidine and uridine pyrophosphates R_F region (0.71–0.77). A trace of guanosine component is also apparent at R_F 0.56 (B) and this is confirmed by a positive fluorescence in the visible region, recorded photographically[8].

Fig. 2. (a), Latex-serum fraction; (b), ADP + ATP; (c), latex-serum fraction (dilute); (d), CDP + CTP; (e), UDP + UTP; (f), latex-serum fraction; (g), ADP + adenosine tetraphosphate; (a)–(d) run at 350 V; (e)–(g) at 250 V.

TABLE I

ULTRAVIOLET-SPECTRAL DATA OF ELUTED SERUM FRACTION COMPONENTS

Spot	λ_{max} *(mμ)*	λ_{min} *(mμ)*
1	261.0	236.0
2	277.5	242.0
3	260.0	235.0
A	258.5	231.5
B	270.0	241.0
C	275.0	241.0

Insufficient of this latter material was present in the samples, however, to enable a more complete identification. In Table I the spectroscopic data, obtained on eluates at pH 1.0, provide additional proof of the presence of cytidine components in spots 2 and C. Close examination of the data also indicates that the two major spots 1 and 2 are mixtures in which ADP and CTP, respectively, predominate. Since no guanosine derivatives are detectable on these electrogram patterns and since uridine derivatives have higher mobilities than spots 1 and 2 it is reasonable to assume the presence of minor amounts of CDP in the latter spot and of ATP in the former, particularly in view of the information obtained from the Dowex-column data.

On this basis the composition of the two-component systems may be calculated in either case by application of the Beer-Lambert law to the absorbancy values at two chosen wavelengths and by use of molar absorbancy data for the four components in question[6]. The result of this calculation is shown in Table II. Spot 3 is calculated as pure UDP since there is little evidence for the presence of a second u.v.-absorbing component.

TABLE II

PYROPHOSPHATE NUCLEOTIDE CONTENT OF LATEX SERUM

	μmoles/g serum solids	*μmoles/l serum*
ADP	0.074	5.2
CDP	0.039	2.8
UDP	0.030	2.1
ATP	0.015	1.0
CTP	0.048	3.4

It is of interest to observe that the total molar concentration of cytidine pyrophosphates in serum is as high as that of the adenosine derivatives, on the present evidence. The major specific function of CDP known at present is as phosphate and choline carrier in the synthesis of phosphatides. It can also transfer phosphate as CTP to mevalonic acid during the formation of isopentenyl pyrophosphate by "MVA kinase"[9].

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Deoxycytidine, deoxyuridine and 5-methyldeoxycytidine in rat urine

The identification of several types of deoxyribosyl compounds which are found in acid extracts of animal tissues has been reported from this and other laboratories (*cf.* ref. 1). As shown by ARIENT *et al.*[2], relatively large amounts of deoxyribosyl compounds are excreted in the urine of rats on a laboratory chow diet. These workers reported deoxycytidine to be the major deoxyribosyl compound excreted by normal animals.

In the course of some experiments which are being carried out in this laboratory to study *in vivo* the metabolism of deoxyribosyl compounds, acid extracts of rat urine were fractionated. In addition to deoxycytidine and 5-methyldeoxycytidine, smaller amounts of deoxyuridine and unidentified deoxynucleotides were found. The following report describes the isolation and identification of these compounds.

Male Holtzman rats were maintained on a purified diet containing, per kg, 180 g "vitamin-free" casein, 50 g corn oil, 40 g salt mixture, 730 g Cerelose, 7.0 mg calcium pantothenate, 3.0 mg thiamin hydrochloride, 2.0 mg riboflavin and 1.0 g choline chloride. The animals were housed in plastic cages which were cleaned and sterilized daily. Daily urinary samples were filtered through glass wool, cooled and acidified to 0.6 *N* with $HClO_4$. After centrifugation the extracts were neutralized with KOH and filtered. In order to remove salts, the neutralized extracts from several collections were concentrated, acidified, and applied to charcoal (Darco-G60)–Celite columns. The adsorbed deoxyribosyl compounds were eluted with ammoniacal ethanol (ethanol–water–28 % aq. NH_3, 50:47:3, v/v/v). The charcoal eluate was evaporated to dryness and the residue was extracted with methanol.

Isolation of the deoxyribosyl compounds was accomplished by paper and ion-exchange chromatography. The purification was followed by assaying sections of the paper chromatograms or aliquots from the column eluates by a modification of the microbiological assay method of HOFF-JORGENSEN[3] using the organism *L. acido-*

TABLE I

R_F VALUES, OPTICAL PROPERTIES AND DEOXYNUCLEOSIDE CONTENT OF NUCLEOSIDES FROM RAT URINE

	Deoxycytidine		*5-methyl deoxycytidine*		*Deoxyuridine*	
	Standard	*From urine*	*Standard*	*From urine*	*Standard*	*From urine*
R_F, Solvent 1*	0.20	0.19	0.24	0.24	0.32	0.33
R_F, Solvent 2	0.60	0.58	0.65	0.63	0.73	0.72
pH 1 A_{280}/A_{260}	2.15**	1.94	3.4**	3.1	0.375**	0.315
Maximum (mμ)	280	280	286	285	262	262
Minimum (mμ)	241	243	245	245	231	230
μg deoxynucleoside calcd. from absorbancy data	—	216	—	43	—	20
μg calcd. from microbiological assay	—	220	—	40	—	21

* Solvents: 1. aq. *n*-butanol, 2. isopropanol–HCl.

** See ref. 4.

philus R-26. The charcoal eluates were chromatographed on Whatman No. 1 paper, descending, first in *n*-butanol–water (86:14, v/v) and then in isopropanol–HCl (isopropanol, 170 ml; conc. HCl, sp. gr. 1.19, 41 ml; water to make 250 ml). Two deoxynucleoside bands and one deoxynucleotide band were obtained. The deoxynucleoside fractions were adjusted to pH 7 and chromatographed on Amberlite XE-64-H$^+$ followed by chromatography on Dowex-1-formate. Like deoxyuridine, one deoxynucleoside fraction was not adsorbed on XE-64 at pH 7 but was adsorbed on Dowex-1 at pH 9.5 and eluted at pH 8. The second deoxynucleoside fraction was adsorbed on XE-64 at pH 7 and eluted with 0.001 N HCl but was not adsorbed on Dowex-1 at pH 9.5. During subsequent repeated chromatography on paper with several different solvents both of these fractions gave only one band but, since the band of the second deoxynucleoside fraction was broad and appeared possibly to contain two components, the leading and trailing edges of this band were eluted separately and observed to have spectra similar to 5-methyldeoxycytidine and deoxycytidine, respectively. Three deoxynucleoside fractions were thus obtained and after further chromatography on paper, these appeared to be reasonably pure on the basis of the properties listed in Table I.

Of the total amount of deoxyribosyl compounds present in the urine extracts, approx. 5 % was accounted for by deoxynucleotides, 10–15 % by deoxyuridine, 60 % by deoxycytidine and 20 % by 5-methyldeoxycytidine.

These studies are being extended to determine the source of the urinary deoxyribosyl compounds.

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Sur la caracterisation des orthoquinones monocycliques au cours de l'oxydation d'acides aminés et amines orthodiphénoliques

Le passage par l'*o*-quinone monocyclique au cours de l'oxydation enzymatique ou chimique des acides aminés et amines *o*-diphénoliques a toujours été considéré comme la seule voie vraisemblable, mais l'instabilité de ces quinones avait gêné leur mise en évidence; quelques preuves expérimentales furent avancées dans le seul cas d'oxydations à pH très bas[1–3]. Ayant observé, au cours de certaines oxydations enzymatiques,

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Abréviations: DNPH, 2,4-dinitrophénylhydrazine; DNPHone, dinitrophénylhydrazone; DOPA, 3,4-dihydroxy-L-phénylalanine.

que le milieu prenait une teinte jaune transitoire, précédant la teinte rose dûe au pigment cyclisé (aminochrome: hétérocycle azoté), nous avons pensé que la quinone dérivant directement du diphénol en était responsable. Nous avons cherché à le démontrer: (a) par la comparaison des caractères spectrophotométriques des milieux selon les conditions d'oxydation; (b) par l'action des réducteurs sur les milieux supposés contenir les quinones: une partie du diphénol disparu est régénéré; (c) par la formation avec la DNPH de composés caractéristiques étroitement apparentés aux DNPHones de quinones connues.

Les substances diphénoliques employées sont des produits commerciaux, sauf l'hydroxyhordénine que nous avons préparée en faisant agir la polyphénoloxydase de champignon[4] sur l'hordénine. Les méthodes d'oxydation (polyphénoloxydase, Ag_2O, PbO_2 en H_2SO_4) et de chromatographie sur papier lavé ont été décrites précédemment ainsi que l'utilisation de la DNPH[5]. Nous employons de l'eau distillée deux fois dans un appareil en verre Pyrex. La spectrophotométrie a été réalisée avec l'appareil enregistreur Beckman DK-2.

Coloration et spectrophotométrie

Une coloration jaune puis jaune-orangé a été observée dans certaines oxydations par la polyphénoloxydase: DOPA (dans l'eau, à 0°) et glycyl-L-tyrosine (à pH 6,8, température ordinaire), pendant quelques secondes; dopamine (dans l'eau, entre 0 et 15°) et glycyltyrosine (dans l'eau, température ordinaire), pendant plusieurs minutes. On trouve alors un maximum d'absorption à 390 mμ (signalé depuis par YASUNOBU pour la glycyltyrosine[6]) qui passe à 385 mμ si l'on ajoute de l'acide sulfurique (normalité finale 1 *N*). Rappelons que les aminochromes ont un maximum vers 305 et vers 480 mμ, en milieu neutre ou peu acide.

Dans les oxydations par PbO_2 en H_2SO_4 1 *N* (DOPA, dopamine, adrénaline, hydroxyhordénine*), la coloration jaune est stable plusieurs heures, mais la spectrophotométrie montre qu'il ne faut pas l'attribuer uniquement à la quinone monocyclique: le pic initial vers 385 mμ s'affaiblit et disparaît (se fondant dans une absorption générale plus ou moins importante) en 20 à 60 min ou plus, selon le diphénol envisagé; en même temps augmente un maximum d'absorption situé entre 258 et 275 mμ selon le cas**. A titre de comparaison, la spectrophotométrie en milieu sulfurique du dopachrome (milieu d'oxydation de DOPA par Ag_2O) et de l'adrénochrome pur fait apparaître un maximum placé à 410 mμ pour le premier, à 405 (après 1 min) puis 395 mμ (après 3 min) pour le second. Les aminochromes sont d'ailleurs détruits en quelques minutes à ce pH. Maximum de l'adrénochrome dans H_2SO_4 concentré: 380 mμ (voir réf. 7).

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* Pour les trois premières, maximum caractéristique entre 382 et 386 mμ; pour l'hydroxyhordénine, à 360–365 mμ (déplacé peut-être vers les faibles longueurs d'onde par l'absorption due à une autre substance).

** La chromatographie sur papier dans l'eau permet d'ailleurs de séparer dans les quatre cas cités un pigment jaune migrant en front avec H_2SO_4, relativement stable, oxydant, non indolique et virant au rose si l'on ajoute du $NaHCO_3$. Les pigments dérivés de la DOPA et de la dopamine ont en milieu acide un maximum d'absorption faible vers 380 mμ et un maximum très important vers 258–265 mμ; après neutralisation par le $NaHCO_3$, maximum à 485 et à 273 mμ. D'autre part, dans le cas de la DOPA, un pigment rose carmin, oxydant et indolique se sépare par chromatographie dans l'eau, juste en deçà de la plage sulfurique. Ce pigment, comme les pigments jaunes neutralisés, vire au gris en peu de temps. Ils pourraient dériver des quinones monocycliques par polymérisation.

Régénération du diphénol initial

Les réducteurs employés sont $NaHSO_3$, KI et le zinc en poudre. Ils sont ajoutés sous forme solide aux milieux d'oxydation à des temps différents. Dans le cas des oxydations par l'enzyme, on dénature au préalable celui-ci par H_2SO_4 pour pouvoir comparer les quantités de diphénol présentes avant et après réduction. La régénération, pour les temps d'oxydation courts, du diphénol par ces réducteurs est observée après chromatographie dans l'eau (cas de la DOPA oxydée par PbO_2, de la dopamine oxydée par PbO_2 ou par la polyphénoloxidase). La réduction par le courant électrique au niveau d'une cathode de platine permet de l'observer par spectrophotométrie. Même en l'absence de réducteurs, une augmentation spontanée de la quantité de diphénol est visible dans les premières minutes suivant la filtration (PbO_2) ou l'acidification (polyphénoloxydase). Ceci peut être dû soit à une dismutation de la quinone, soit à sa réduction par un produit ultérieur.

Action de la DNPH

Les précipités lavés obtenus après action de la DNPH sur les milieux d'oxydation sont étudiés par chromatographie sur papier et par spectrophotométrie. L'analyse chromatographique sépare plusieurs produits colorés; dans tous les essais où nous soupçonnons la présence d'une quinone monocyclique et dans ceux-là seulement, une tache prédominante, initialement orangé-brique, vire au bleu ou au violet lorsque le chromatogramme est exposé à des vapeurs d'ammoniac. C'est le cas de la *p*-benzoquinone, du 1,2-naphtoquinone 4-sulfonate de sodium, du catéchol et des 3- et 4-méthylcatéchols oxydés par Ag_2O (temps d'oxydation très court pour avoir presque exclusivement l'*o*-benzoquinone[8]) ou PbO_2, des DOPA, dopamine, adrénaline et hydroxyhordénine oxydées par PbO_2, des glycyltyrosine et dopamine oxydées par la polyphénoloxydase.

Nous avons élué ces produits caractéristiques (DNPHones probables) et étudié leur spectre à différents pH, ainsi que celui des précipités lavés eux-mêmes (*cf.* tableau). La comparaison des spectres à pH acide permet de distinguer trois groupes: A: maximum à 450–456 mμ; épaulement à 391 mμ: là où l'aminochrome est prédominant. B: maximum vers 345 mμ; épaulement ou maximum peu marqué vers 410 mμ: là où on attend une grande quantité de quinone monocyclique. C: maximum entre 340 et 360 mμ et maximum entre 430 et 450 mμ. Le produit qui vire au bleu-violet par l'ammoniac isolé des groupes B ou C, donne un spectre de type B. Le type C doit donc être formé par juxtaposition à la DNPHone de la quinone monocyclique, de la DNPHone de l'aminochrome (oxydation par la polyphénoloxydase) ou d'un corps inconnu (DOPA oxydée par PbO_2). En milieu alcalin on note: maximum principal entre 580 et 598 mμ là où existe l'aminochrome; entre 559 et 569 mμ là où domine la quinone monocyclique.

L'ensemble de ces résultats peut s'interpréter par la présence d'une *o*-quinone monoclyclique responsable de l'absorption à 385 ou 390 mμ, de la régénération de l'*o*-diphénol, et de la formation d'une DNPHone nettement différente de celle des aminochromes. A l'appui de cette hypothèse, on peut noter que ces trois propriétés des milieux d'oxydation disparaissent après des temps du même ordre bien qu'il soit impossible de faire une cinétique précise: l'existence d'une absorption générale croissant avec le temps ne permet pas un dosage spectrophotométrique. En ce qui

TABLEAU I

Caractères spectrophotométriques des précipités obtenus avec la DNPH. (M, Maximum; PM, Maximum peu marqué; E, Epaulement). Les résultats varient peu quand on passe de pH 3 à pH 5 et de pH 9 à pH 13.

Précipités (lavés, puis dissous dans l'alcool) obtenus avec:	*pH acide (HCl)*			*pH alcalin (NaOH)*	
	340–370 mμ	*370–440 mm*	*450–460 mμ*		
Adrénochrome pur + DNPH		E 391	M 456	E 465	M 598
Adrénaline + Ag_2O + DNPH		E 391	M 453	E 460	M 598
DOPA + Ag_2O + DNPH		E 391	M 450	M 460	M 580
DOPAmine + polyphénoloxydase + DNPH					
(1° préparation	M 355		M 450	M 373	M 580
(2° préparation (I)	M 345	M 430		M 373	M 568
DOPAmine + DNPH + polyphénoloxydase (II)		M 382	M 450	PM 432	M 595
Glycyltyrosine + polyphénoloxydase + DNPH (III)	M 353	PM 422		...	M 565
DOPAmine + PbO_2 + DNPH	M 346	E 409		M 370	M 565
Adrénaline + PbO_2 + DNPH (IV)	M 345	PM 408		M 360	M 562
DOPA + PbO_2 + DNPH (V)	M 368	M 431		M 384	M 562
Catéchol + Ag_2O ou PbO_2 + DNPH	M 343	PM 410		M 365	M 560
4-méthylcatéchol + Ag_2O ou PbO_2 + DNPH	M 356	PM 420		M 374	M 569
3-méthylcatéchol + Ag_2O ou PbO_2 + DNPH	M 358	PM 435		M 385	M 585
p-Benzoquinone + DNPH		M 393		E 365	M 551
DNPHone isolée de (I)	M 346	E 408		M 370	M 560
DPNHone isolée de (II)	M 339	E 409		E 370	M 565
DPNHone isolée de (III)	M 341	E 420		...	M 565
DPNHone isolée de (IV)	M 346	E 408		PM 365	M 559
DPNHone isolée de (V)	M 346	E 419		M 370	M 565

concerne la régénération du diphénol, il faut admettre que les moyens de caractériser celui-ci ne sont pas absolument spécifiques, et on peut craindre la présence simultanée d'un composé de même R_F en chromatographie ou absorbant dans la même zone en spectrophotométrie. Par contre l'action de la DNPH a [illegible]nné des résultats très concordants pour les oxydations par PbO_2 et par la polyphénoloxydase, avec des produits tout à fait comparables aux DNPHones de quinones connues. C'est l'argument principal pour déterminer la nature du composé transitoire révélé par la spectrophotométrie.

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Enzymic hydrolysis of uridine diphosphate glucuronate in rat skin

Uridine diphosphate glucuronic acid when added to rat-skin extracts was found to disappear rapidly. A study of the products formed in this reaction has shown the presence of soluble enzymes which catalyze the following reactions:

$$\text{UDPGA} \xrightarrow{\text{pyrophosphatase}} \text{UMP} + \text{GA-1P}$$

$$\text{UMP} \xrightarrow{\text{phosphatase}} \text{Uridine} + \text{phosphate}$$

$$\text{GA-1P} \xrightarrow{\text{phosphatase}} \text{Glucuronate} + \text{phosphate}$$

A similar kidney particulate system was described recently[1].

UDPGA (NH^+_4, 90 %) (Sigma Chemical Co.). GA-1P was prepared by chemical oxidation of glucose-1-phosphate[2]. *p*-Nitrophenol was recrystallized from ethanol–water.

UDPGA was measured by a modification of the procedure of ISSELBACHER[3] for following *p*-nitrophenol glucuronidation. Guinea-pig liver microsomes were prepared by homogenizing the tissue from exsanguinated animals in 4 vol. 0.154 *M* KCl, the precipitate obtained after 10 min centrifugation at 2,000 × *g* was discarded, and the supernatant fluid was centrifuged 90 min at 18,000 × *g*. The precipitate was suspended in an equal volume of KCl solution and centrifuged 60 min at 18,000 × *g*. The washing was repeated and finally the precipitate was resuspended to the original volume in KCl. The activity of such preparations was stable for several weeks when stored at —15°.

UDPGA was assayed by incubating the following reaction mixture: 0.01 ml 1.7 m*M* *p*-nitrophenol in 0.5 *M* phosphate buffer (pH 7.4), 0.04 ml of rehomogenized microsomes and UDPGA in a final volume of 0.1 ml. All mixtures were assayed in duplicate and controls without UDPGA were always included. Incubation was carried out with shaking at 37°, and the time was dependent upon microsomal activity, 15–30 min being usually sufficient with fresh microsomes. The reaction was stopped by the addition of 0.4 ml 0.1 *N* trichloroacetic acid, and the color measured at 400 mμ after addition of 0.01 ml 10 *N* KOH to the supernatant. The decrease in absorbancy was a linear function of the UDPGA concentration when it did not exceed 0.3. Amounts of UDPGA between 0.002 to 0.02 μmole could be determined with the method.

For measurement of UDPGA disappearance, UDPGA plus the skin enzyme were incubated in a volume of 0.05 ml and the reaction stopped by placing the tubes in boiling water for 1 min. Microsomes and *p*-nitrophenol were then added for the second incubation. Controls with boiled skin enzyme were always included.

Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[4] in a final volume of 0.5 ml, uronic acid by the carbazole reaction[5], and reducing sugar by the PARK-JOHNSON method[6]. Nucleotides were located visually on paper chromatograms with an u.v. lamp and sugars revealed by reduction of Ag^+ (see ref. 7).

Abbreviations: UDPGA, uridine diphosphate glucuronate; UDPAG, uridine diphosphate N-acetyl-glucosamine; UDPG, uridine diphosphate glucose; UMP, uridine monophosphate; GA-1P, α-D-glucuronate-1-phosphate; EDTA, ethylenediaminetetraacetate.

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In order to reveal GA-1P, 1.5 *N* NaOH in ethanol was used in place of 0.5 *N* NaOH and the paper chromatograms were heated over a steam bath.

Skin was removed from exsanguinated young rats (7 to 14 days old), cut into small pieces, and ground in a mortar in 2 vol. 0.154 *M* KCl/g of tissue. Some preparations were extracted in the squeezing device of FRIEDENWALD AND MOSES[8] and were equally active. After filtration through cheese-cloth, the extract was centrifuged 30 min at 10,000 × *g* and the supernatant decanted and dialyzed overnight against distilled water. All the procedures were carried out at 0–4°. The dialyzed enzyme was stable for at least 3 weeks when stored at —15°.

Homogenates of rat skin catalyzed the rapid disappearance of UDPGA at pH 7.4 and 37°. Both the 10,000 × *g* precipitate and supernatant catalyzed this disappearance and UDPAG inhibited the breakdown in all fractions (67–86 % with UDPAG at 1.4 m*M*, UDPGA at 0.3 m*M*, and 30-min incubation). The activity of the precipitate was inhibited similarly by UDPG. The supernatant was used in the remainder of the present studies. Both UDPGA disappearance and GA-1P phosphatase activities consistently were increased after dialysis. High UDPGA disappearance activities were regularly obtained, but GA-1P-ase activity was variable, often being very low. EDTA at 0.01 *M* completely inhibited UDPGA breakdown, and Mg^{++} had no effect on the enzyme, even after overnight dialysis against 0.001 *M* EDTA. Since exhaustive dialysis had no effect on UDPGA disappearance, it appeared most probable that the enzyme involved was a pyrophosphatase, rather than a pyrophosphorylase which would have required inorganic pyrophosphate.

After incubation, removal of proteins with $HClO_4$, and of nucleotides by charcoal adsorption, an increase was found in both non-nucleotide glucuronate and reducing power after acid hydrolysis. Paper chromatography was used further to establish the nature of the products. Incubations usually were carried out with the reaction mixtures adjusted to pH 9 with ammonia and no buffer added. After 6–7 h at 37°, the samples were deproteinized with trichloroacetic acid and the latter was removed with ethyl ether. Upon chromatography in ethanol–1 *M* ammonium acetate (75:30) (see ref. 9), spots corresponding to UMP, uridine, GA-1P, glucuronate and glucuronolactone were found. A typical chromatogram is shown in Fig. 1. The UMP was not always found, and high UMP-phosphatase activity was found in these enzyme preparations. All of the compounds except UMP were tested and found to move identically with standards in ethanol–1 *M* ammonium acetate (pH 3.8) (75:30) (see ref. 9). No measurable lactone accumulation could be measured by the hydroxamate method[10], and the glucuronolactone probably was formed during chromatography, as standard glucuronate also formed two spots.

The skin extracts also were found to degrade several nucleotide-sugar compounds. After incubation at pH 9 with either UDPAG, UDPG, or guanosine diphosphate mannose, new spots appeared on chromatography corresponding to hexose phosphates and either uridine with the former two or guanosine monophosphate and guanosine with the latter.

Inorganic phosphate was released more rapidly at pH 9 in potassium–glycine buffer from GA-1P than at pH 7 in potassium–tris(hydroxymethyl)aminomethane-maleate buffer. EDTA inhibited this reaction by 67 % at 0.001 *M* and by 98 % at 0.01 *M*. Although other metal chelators also inhibited the phosphatase (27 % inhibition by 0.001 *M* 8-hydroxyquinoline, 21 % by 0.001 *M* α,α′-dipyridyl), no activation was found with any metal. At 0.001 *M*, the following cations were inhibitory: Zn^{++}, Ni^{++}, Co^{++}, Cu^{++}, Ca^{++}, Mg^{++}, Mn^{++}, Fe^{++}, and Fe^{+++}.

Spots corresponding to glucuronate and its lactone were found upon chromatography after enzyme action. In addition, a new reducing spot which ran between GA-1P and glucuronate in ethanol–1 *M* ammonium acetate (75:30) appeared when either UDPGA or GA-1P were incubated with enzyme at pH 9. This spot was not observed when either phosphate or glucuronate alone plus enzyme were incubated, but a similar spot appeared after incubating phosphate and glucuronate alone. Apparently this spot was formed by a non-enzymic reaction and it was not investigated further.

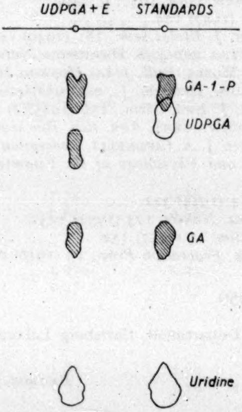

Fig. 1. Paper chromatography (descending) of metabolic products of UDPGA.

The major route for ascorbic acid biosynthesis is now considered to be from glucuronate[11], and the enzymes described in this paper provide a metabolic pathway for the formation of this intermediate in skin. It is of interest that UDPGA also is an important intermediate in mucopolysaccharide formation[12]. The well established primary effects of vitamin C deprivation in connective tissues[13] may be related to the necessity for UDPGA in both pathways. Several investigators have reported a decreased formation of acid mucopolysaccharides in the cartilage and skin of scorbutic guinea pigs[14–17]. If the necessary enzymes for ascorbate formation from glucuronate occur in connective tissue, a control mechanism may exist so that in the presence of normal ascorbate levels, sufficient UDPGA is channeled into mucopolysaccharide formation. With a deficiency of the vitamin, the UDPGA might have to serve mainly for glucuronate and ascorbate formation. A possible mechanism for this control could be a negative-feedback effect, *i.e.* inhibition by ascorbate of one of the enzymes in the sequence UDPGA → ascorbate.

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Studies on the acid-insoluble fraction of Frog's muscle

In the study of muscular contraction, interest has centered upon the participation of the phosphoryl groups of the acid-soluble components of muscle in this process. There is no report on the possible changes of the phosphoryl groups of the acid-insoluble components of muscle, accompanying muscular activity.

It was of interest, therefore, to investigate this problem, particularly because of the reports regarding phosphate transfer from phosphate-enriched RNA to fructose monophosphate[1] and a reversible transphosphorylation, in the presence of creatine phosphokinase, to creatine[2]. Furthermore, nucleic acid was found to be associated with myosin preparations[3] and its presence in myofibrils was demonstrated recently[4].

Gastrocnemius and sartorius muscles of *Rana catesbiana* and the sartorius muscles of *Rana pipiens* were used. Two techniques were employed for the stimulation:

1. The gastrocnemius muscles of both legs were exposed and the central end of the sciatic nerves were cut. One nerve was stimulated for 40 sec at 10V with a

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; Tris, tris(hydroxy methyl)aminomethane.

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frequency of 20 pulses/sec and pulse duration of 1 msec. The other nerve-muscle preparation served as a control. Both muscles were excised and frozen by immersion into isopentane at —180°, at the time when the stimulated muscle was still contracted. In these experiments it was impossible to eliminate a small contraction of the control gastrocnemius on freezing, and for this reason the sartorius muscles were used in later experiments.

2. The sartorius muscles of both legs were dissected out and allowed to recover in frog's Ringer solution, under oxygen, for 30 to 60 min. The muscles were then mounted on a multi-electrode stimulator[5], at 0.5°–2°, in an atmosphere of nitrogen saturated with water vapor, afterloaded with approximately 10 g/100 mg muscle.

TABLE I

THE DISTRIBUTION OF PHOSPHATE IN THE ACID-INSOLUBLE FRACTION OF FROG'S MUSCLE

Muscle	*Total acid-insoluble µmoles P/g muscle*	*RNA fraction µmoles P/g muscle*	*DNA µmoles P/g muscle*	*RNA µmoles P/g muscle*	*Inorganic P µmoles/g muscle*
Rana catesbiana					
Gastrocnemius					
Control	2.6	1.74	0.86	1.38	0.3
Stimulated	2.6	1.82	0.78	1.37	0.3
Sartorius					
Control	3.22	1.97	1.25	0.82	0.9
Stimulated	3.12	1.97	1.15	0.81	0.9
Rana pipiens					
Sartorius					
Control	3.16	1.84	1.32	1.12	0.6
Stimulated	3.09	1.84	1.19	1.11	0.7

Following 10 min of equilibration, one muscle was stimulated for approximately 20 sec at 4V with a frequency of 20 pulses/sec and pulse duration of 2 msec. Both muscles were quick frozen in isopentane at —180°. Larger muscles were used directly for analysis, while the sartorius muscles of *Rana pipiens* were kept frozen in liquid nitrogen until at least five pairs of muscles were pooled. There was no visible contraction on freezing of the control muscle, as indicated in Fig. 1.

The muscles were ground in liquid nitrogen and weighed into beakers containing cold 10 % trichloroacetic acid. A modified procedure of SCHMIDT AND THANNHAUSER[6] was used for the fractionation of the acid-insoluble components into the RNA-containing fraction and the DNA-containing fraction. The fractionation method of SCHNEIDER[7] was used in some experiments and the method of HUGGINS AND COHN[8] was used for the determination of phospholipid and phosphatido peptide phosphate. Phosphate was determined according to the method of FISKE AND SUBBAROW[9] and for aliquots containing less than 0.4 µmole P, the method of BERENBLUM AND CHAIN[10] was used.

The DNA was extracted from the DNA-containing fraction with 0.5 *N* $HClO_4$ at 70° (see ref. 11), and 90–100 % of the total P of this fraction was accounted for by the DNA-P.

The RNA-P was determined after further fractionation of the RNA-containing fraction. Following the removal of perchlorate with KOH, 0.1 M Tris buffer, pH 7.5, was added to an aliquot to a final concentration of 0.025 M. The mixture was placed on Dowex 2, Cl^- form column. The bulk of the u.v.-absorbing material, presumably protein breakdown products, was not absorbed on the resin and the mononucleotides and inorganic P were eluted with 1 N HCl. An aliquot of the acid eluate was taken for P analysis and the remaining solution was lyophilyzed and chromatographed in isopropanol–HCl solvent system[12]. Four spots characteristic of RNA were obtained, as shown in Fig. 2. The molar ratio of adenine : guanine : cytosine : uracil was 1:1.25:1.1:1.03, respectively.

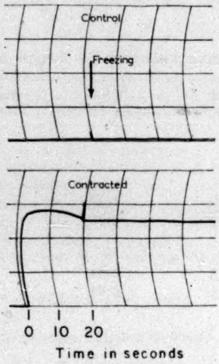

Fig. 1. Sartorius of *Rana pipiens*, control and stimulated, quickfrozen at arrow.

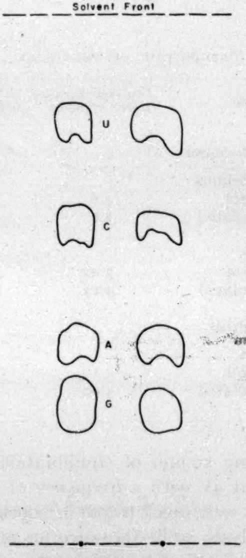

Fig. 2. Paper chromatography of acid hydrolysates of muscle RNA.

The distribution of P of the acid-insoluble components of frog's muscle is shown in Table I, for the control and the stimulated muscle. The total acid-insoluble P, the DNA-P and the RNA-P were identical in the control and in the stimulated muscle. The inorganic P derived from phosphoprotein was not altered upon stimulation. There was no significant difference between the control and the stimulated muscle in either the phospholipid or the phosphatido peptide P, as shown in Table II.

A change of 0.5 μmole P/g tissue has been quoted as the minimum change expected in a single muscle twitch[13], if the compound under consideration participates in contraction. Such change would have been easily detected with the methods used in this investigation, considering that the total acid-insoluble P is between 2.6 and 3.2 μmole P/g muscle. None of the components studied was significantly altered during contraction and it is reasonable to conclude that the phosphoryl groups of the acid-insoluble compounds of muscle are not involved in the process of contraction. While this study was in progress, **Abood, Goldman and Lipman**[14] had published their findings on frog's nerve during excitation. The results were similar to the ones on muscle, where neither the DNA-P, nor the RNA-P altered significantly and the phospholipid and phosphatido peptide P did not change.

TABLE II

THE PHOSPHOLIPID AND PHOSPHATIDO PEPTIDE P IN FROG'S MUSCLE

	Phospholipid P µmoles/g muscle	*Phosphatido peptide P µmole/g muscle*
Control	0.35	0.75
Stimulated	0.36	0.9
Control	0.43	0.88
Stimulated	0.38	0.79

We would like to thank Dr. B. C. **Abbott** and Dr. W. H. F. M. **Mommaerts** for their interest in this work.

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Studies on the biosynthesis of uroporphyrin III from porphobilinogen and the behaviour of uroporphyrin esters in paper chromatography

It has recently been demonstrated that two enzymes are involved in the biosynthesis of uroporphyrinogen III from porphobilinogen[1,2]. One of these enzymes, porphobilinogen deaminase, catalyses the condensation of 4 molecules of porphobilinogen to uroporphyrinogen I; the second enzyme, uroporphyrinogen isomerase, has no effect upon porphobilinogen when incubated alone with it and, moreover, is unable to convert uroporphyrinogen I to uroporphyrinogen III. However, both of these enzymes together are required for the formation of uroporphyrinogen III.

Kinetic data suggest that there is, at some point in the biosynthesis of uroporphyrinogen III, a direct interaction between porphobilinogen and the uroporphyrinogen isomerase. This suggested the possibility that uroporphyrinogen isomerase might catalyze an exchange reaction between uroporphyrinogen I and porphobilinogen (activated in some manner by porphobilinogen deaminase) to give uroporphyrinogen III. In order to test this hypothesis 5.74 μmoles porphobilinogen were incubated anaerobically with porphobilinogen deaminase, uroporphyrinogen isomerase[2], and ^{14}C-labelled uroporphyrinogen I, prepared by sodium amalgam reduction of 0.76 μmole of ^{14}C-labelled uroporphyrin I*. If uroporphyrinogen I were an intermediate in the biosynthesis of uroporphyrinogen III, ^{14}C-labelled uroporphyrinogen III should have been produced in this experiment. Consequently after the porphobilinogen had been consumed, the uroporphyrins were isolated from the reaction mixture and esterified and a separation of the uroporphyrin isomers was attempted to ascertain whether the uroporphyrin III was labelled with ^{14}C.

Initially we investigated the possibility of using the paper-chromatographic procedure of FALK AND BENSON[3] to separate the two isomers on a preparative scale. For these investigations ^{14}C-labelled uroporphyrins I and III were synthesized enzymically[1,2] from [^{14}C]porphobilinogen. The [^{14}C]uroporphyrin III octamethyl ester was homogeneous on paper chromatography as judged by fluorescence and radioautography. Traces of porphyrin were found at the uroporphyrin III position on the paper when a sample of the [^{14}C]uroporphyrin I octamethyl ester was chromatographed; authentic uroporphyrin I behaved similarly. The following mixtures of uroporphyrins I and III were then prepared for chromatographic analysis: (a) a 1:1 mixture of [^{14}C]uroporphyrin I octamethyl ester, and (b) a 5:4 mixture of [^{14}C]uroporphyrin III octamethyl ester and unlabelled uroporphyrin I octamethyl ester. Aliquots of 0.26 μg porphyrin from mixture (a) were applied, 15 per sheet, to 10 sheets of Whatman No. 1 filter paper. After development of the chromatograms the spots at the uroporphyrin I and III positions were cut out separately and the porphyrins were eluted with chloroform and collected. A similar procedure was followed with aliquots of mixture (b). The porphyrin isomers were purified by extraction from an ether solution with 2 N HCl and immediate retransfer to fresh ether by adjusting the pH of the aqueous solution 4. The activity of the porphyrins was measured at infinite thinness in a windowless gas-flow counter (efficiency 39 %). The results, summarized in Table I, show clearly that no separation of the uroporphyrin I and III isomers resulted. Since these isomers when chromatographed singly behaved essentially as single components, these results indicate that some type of association occurs between the uroporphyrin isomers so that when mixed and then chromatographed they do not behave as they do when chromatographed alone. To our knowledge this type of behaviour in paper chromatography has not been observed previously. The FALK AND BENSON method remains, however, a very useful analytical tool.

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* Porphobilinogen, having $1.27 \cdot 10^5$ counts/min/μmole, was prepared from [5-^{14}C]-5-aminolevulinic acid by a purified enzyme fraction from chicken erythrocytes essentially according to the procedure of R. SCHMID AND D. SHEMIN (*J. Am. Chem. Soc.*, 77 (1956) 506) for duck erythrocytes The [^{14}C]porphobilinogen was in turn converted to [^{14}C]uroporphyrin I by a preparation of porphobilinogen deaminase.

TABLE I

THE SPECIFIC ACTIVITIES OF THE UROPORPHYRIN ISOMERS AFTER SEPARATION BY FALK AND BENSON CHROMATOGRAPHY

Expt.	*Constitution of porphyrin mixture applied*	*Specific activity of labelled uro ester counts/min/μg*	*Amount of porphyrin eluted (μg)*		*Specific activity of porphyrin eluted counts/min/μg*	
			Position on chromatogram			
			Uro I Ester	Uro III Ester	Uro I Ester	Uro III Ester
1	[^{14}C]Uro I ester + Uro III ester (ratio 1:1)	700	4.53	10.32	303	252
2	[^{14}C]Uro III ester + Uro I ester (ratio 5:4)	700	4.23	10.08	212	214

Results similar to the above were obtained when the behaviour of mixtures of ^{14}C-labelled and unlabelled coproporphyrin I and III methyl esters was studied using the paper chromatographic method of GRANICK AND MAUZERALL[4].

We next investigated the procedure of CHU AND CHU[5] for the separation of the uroporphyrin I and III isomers on columns of Hyflo SuperCel. A mixture of 250 μg uroporphyrin III ester and 180 μg ^{14}C-labelled uroporphyrin I ester (700 counts/min/μg) was chromatographed. The entire fluorescent zone, measuring approx. 15 cm, was extruded from the column and cut into fifteen 1-cm portions. The porphyrin in the 5 portions from the top of the fluorescent zone was found to have an average specific activity of approx. 100 counts/min/μg; it was therefore mainly uroporphyrin III ester. These 5 porphyrin fractions were combined and re-chromatographed on a small column (0.75 × 40 cm). The fluorescent zone was extruded and a 1-cm fraction cut from a little below the top of the fluorescent zone. The porphyrin eluted had a specific activity of 16.5 counts/min/μg and so was essentially uncontaminated with [^{14}C]uroporphyrin I ester.

Since this method was suitable for obtaining a specimen of uroporphyrin III ester from a mixture of the isomers, the uroporphyrin mixture obtained in the enzymic reaction was resolved by the above procedure. From the top of the fluorescent zone of the 2nd column a uroporphyrin III ester fraction was obtained having 18 counts/min/μg. This is comparable to the level of activity obtained in the control experiment with unlabelled uroporphyrin III ester.

Within the limits of the experimental error of this method it is therefore concluded

that uroporphyrinogen I is not an intermediate in the biosynthesis of uroporphyrinogen III.

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Purification de la lipase à partir du suc pancréatique de porc

Nous avons décrit dans deux récents mémoires[1,2] les principales étapes de la purification de la lipase pancréatique de porc à partir de la pancréatine. Ces étapes sont les suivantes: Extraction aqueuse de la pancréatine, précipitation fractionnée des extraits par $(NH_4)_2SO_4$ et l'acétone, adsorption sélective de la lipase sur phosphate tricalcique, élimination de quelques impuretés par l'alumine $C\gamma$ et enfin électrophorèse de zone à pH 5.25. Les préparations ainsi obtenues possèdent de façon reproductible une activité spécifique de 3500–3600. Elles se comportent comme des préparations homogènes au cours d'une nouvelle électrophorèse à pH 5.25 ou pH 8.0 et au cours d'une chromatographie sur phosphate tricalcique.

Il est alors intéressant de chercher à purifier la lipase à partir du suc pancréatique de porc afin de savoir si l'enzyme véhiculé *in vivo* par le suc est bien le même que celui isolé de la pancréatine et si la même activité spécifique maximum peut être atteinte dans les deux cas. Une telle limite commune représenterait en effet un excellent critère de pureté pour nos préparations, puisque les protéines accompagnant la lipase dans le suc et dans les extraits de pancréatine sont différentes.

Sur un porc de 65 kg, à jeun depuis 48 h et anesthésié au pentothal, on établit une fistule pancréatique permanente[*] permettant l'écoulement normal du suc dans le duodénum en dehors des périodes de collection (2 fois 2 h chaque jour). Une opération bien réalisée permet de collecter 150–300 ml de suc par jour pendant 3 semaines, soit 4–5 l au total. Le suc est lyophilisé immédiatement et conservé à —20° dans des flacons étanches.

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[*] L'opération qui, semble-t-il, n'avait pas encore été pratiquée sur le porc, consiste à enfoncer par l'extérieur une première sonde dans le canal de Santorini et une deuxième sonde un peu plus large dans le duodénum. Nous tenons à remercier très vivement le Professeur MARCENAC et le Docteur LEROY du Service de Chirurgie Expérimentale de l'Ecole Vétérinaire d'Alfort qui ont réussi brillamment cette opération, ainsi que le Professeur FERRANDO, Directeur de l'Ecole, pour son amicale coopération.

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On dissout dans un tampon pH 8.0 0.05 M en tris(hydroxyméthyl)aminométhane et 10^{-3} M en diisopropylfluorophosphate une quantité suffisante de poudre lyophilisée pour que la concentration des protéines soit 2 % environ. Après avoir précipité et lavé les protéines insolubles dans $(NH_4)_2SO_4$ 0.50 saturation, on fractionne ces protéines entre 0.20 et 0.40 saturation. On dissout la fraction (concentration des

TABLEAU I

PURIFICATION DE LA LIPASE A PARTIR DU SUC PANCRÉATIQUE ET A PARTIR DE LA PANCRÉATINE

Etapes de la purification	*Suc pancréatique*		*Extraits aqueux de pancréatine*	
	Activité spécifique	*Rendement %*	*Activité spécifique*	*Rendement (%)*
Etat initial	65–70	100	50–60	100
Précipitation par $(NH_4)_2SO_4$	100	80	100–120	70
Précipitation par l'acétone et traitement à pH 6 (C_3)	250–350	70	500–550	50
Adsorptions (C_5)	750–800	30	1100–1200	25
Electrophorèse	3430	15	3500–3600	20

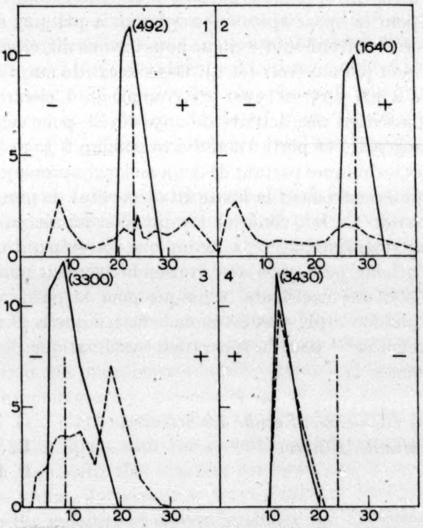

Fig. 1. Electrophorèse de la lipase du suc pancréatique de porc. 1. Suc pancréatique non purifié (activité spécifique (AS): 65–70). Tampon pH 5.25, 0.025 M en acétate et 0.005 M en $CaCl_2$. Gradient: 9V/cm. Durée: 21 h. 2. Préparation C_3 (AS: 250). Même tampon que pour 1. Gradient: 7.0V/cm. Durée: 47 h. 3. Préparation C_5 (AS: 750). Même tampon que pour 1, mais sans calcium. Gradient: 9.9V/cm. Durée: 42 h. 4. Mélange des meilleures fractions du diagramme 3. (AS: 2850). Tampon pH 8.0, 0.025 M en véronal et 0.005 M en $CaCl_2$. Gradient: 7.4 V/cm. Durée: 24 h. Conformément à nos conventions habituelles, les ordonnées correspondent aux pourcentages par ml d'éluat (calculés à partir des quantités introduites dans la colonne) de la lipase (trait plein) et des protéines totales (trait pointillé). Les chiffres entre parenthèses indiquent l'activité spécifique de la fraction la plus pure. Le trait vertical en pointillé matérialise la position initiale, compte tenu du flot électroosmotique.

protéines: 2 % environ) dans un tampon acétate 0.05 *M* pH 5.3 en vue de la précipitation acétonique. Au delà de ce point, on utilise exactement la même technique que pour la pancréatine[2]. Les résultats obtenus sont donnés dans le Tableau I.

La Fig. 1 d'autre part indique les résultats de 4 électrophorèses[1,2] réalisées sur le suc entier et sur des fractions de ce suc purifiées selon les indications du Tableau I.

L'examen du Tableau I et de la Fig. 1 suggère les quelques remarques suivantes:

1. Le schéma conçu pour la purification de lipase à partir de la pancréatine s'applique sans difficulté au suc. Sous réserve de quelques modifications minimes*, imposées sans doute par la différence de composition des mélanges traités, les mêmes opérations permettent d'atteindre dans les deux cas des taux de purification voisins, avec des rendements comparables. En outre, les deux enzymes manifestent un comportement électrophorétique analogue. Ils sont à peu près isoélectriques à pH 5.25 dans un tampon acétate 0.05 *M* (diagrammes 1–3) et migrent de la même façon vers la cathode à pH 8.0 dans un tampon véronal 0.025 *M* (diagramme 4). Tout laisse donc supposer qu'ils sont identiques.

2. Diverses préparations de lipase obtenues sous une forme de plus en plus purifiée à partir de pancréatine et à partir de suc ont été soumises à des électrophorèses de longue durée. Dans le cas des opérations partant de la pancréatine, l'activité spécifique maximum obtenue pour la lipase après électrophorèse à pH 5.25 est 3500–3600. Les fractions douées de cette activité sont, comme nous l'avons dit, électrophorétiquement homogènes à pH 5.25 et pH 8.0 (voir ref. 2). Dans le cas du suc, l'activité maximum après électrophorèse à pH 5.25 est 3300 (diagramme 3). L'électrophorèse à pH 8.0 d'une préparation possédant une activité de 2850 révèle pour cette préparation un état voisin de l'homogénéité et porte l'activité maximum à 3430 (diagramme 4). En d'autres termes, deux techniques partant de deux mélanges protéiques essentiellement différents amènent progressivement la lipase au même état de pureté qu'il ne semble pas possible de dépasser. Ce fait confirme l'impression laissée par les tests électrophorétiques et chromatographiques[1,2], à savoir que les préparations de lipase dont l'activité spécifique atteint 3500–3600 sont vraisemblablement pures.

3. Bien qu'elle soit une excellente technique pour la purification de la lipase, l'électrophorèse sur amidon à pH 5.25 est moins efficace que la chromatographie sur diéthylaminoéthyl-cellulose[3,4] pour la séparation systématique des enzymes du suc pancréatique (diagramme 1).

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* Fractionnement par $(NH_4)_2SO_4$ entre 0.20 et 0.40 saturation (au lieu de 0.25–0.45 saturation); dissolution de cette fraction dans un tampon acétate 0.05 *M* à pH 5.3 (au lieu d'une solution 0.09 *M* de $(NH_4)_2SO_4$).

The lipid composition of human bile

The controversial nature of much of the earlier work on the lipid composition of human bile is attributable largely to the inadequacy of the methods employed. Phospholipid and cholesterol have long been known to be bile constituents, but a more detailed analysis has had to await the development of improved techniques. In 1951, ISAKSSON[1] showed that the molar phosphorus:choline ratio of a lipid extract of human gall-bladder bile was close to one, indicating that essentially all of the phospholipids were present as choline compounds. In addition, he found that all of the phosphoric acid of this extract could be liberated by treatment with KOH, suggesting to him that sphingomyelin was not present in more than insignificant amounts. He concluded, therefore, that lecithin was the only phospholipid in human bile. The same conclusion was reached by POLONOVSKI AND BOURRILLON[2] from analysis of phospholipid fragments obtained on hydrolysis.

In the present investigation, chromatography on silicic acid columns and on filter paper impregnated with silicic acid was used for the separation, identification, and quantitation of the bile lipids. Samples of bile were collected over an approximately 2-h period from the common bile duct T-tubes of six patients who had had cholecystectomies and common bile-duct explorations for gall-stones. The extraction procedure, using chloroform–methanol (1:1), and the chromatographic, staining, and analytical chemical procedures were identical to those employed for serum in previous studies[3,4]. Quantitation of the individual phospholipids was accomplished by measuring the phosphorus in the fractions eluted from the column. Triglyceride was calculated as one-third the difference between total non-phosphorus-containing lipid ester and cholesterol ester.

As can be seen in Table I, lecithin was found to comprise almost all of the phospholipid of human bile. Small amounts of phospholipid had the mobility of "cephalin" and lysolecithin. In one patient, however, approximately 3 % of the total phospholipid had the mobility of lysolecithin, the identity of which was confirmed on paper chromatography. An average of 7.7 % of the total phospholipid migrated on the column as sphingomyelin, but from the shape of the elution curve and the absence of a corresponding spot with the mobility of sphingomyelin on paper chromatography, it was concluded that this phosphorus fraction represented the tail of the lecithin peak.

On paper chromatography, essentially all of the material staining with the phosphomolybdic acid method migrated as a single spot with the mobility of serum lecithin (Fig. 1). Faint spots with the mobility of serum lysolecithin and sphingomyelin were also seen with this staining reaction, and it is concluded that these phospholipids are ordinarily present in trace amounts. Ninhydrin staining revealed a faint spot with the mobility of serum "cephalin" in the three samples tested.

Whether esterified cholesterol and triglyceride are normal constituents of bile has been controversial, but the present investigation suggests the presence of both.

Thus, lecithin and free (unesterified) cholesterol appear to comprise almost all of the lipid of human hepatic bile. The average molar ratio of free cholesterol:lecithin of bile of 0.52 found in this study is quite similar to the average ratio of 0.58 found in normal serum[5], although much individual variation was seen (Table I), suggesting a possible complex between these two substances. ISAKSSON[6], in fact, has provided evidence for complex formation between lecithin, bile salts, and cholesterol.

TABLE I

LIPID COMPOSITION OF HUMAN BILE AS OBTAINED BY CHROMATOGRAPHY ON SILICIC ACID COLUMNS

Patient	*Bile flow*	*"Cephalin"*		*Lecithin*		*Lysolecithin*		*Total lipid P*	*Recovery*	*Free cholesterol*	*Total cholesterol*	*% free*	*Total non-P ester*	*Triglyceride*
	ml/h	%	*mM*	%	*mM*	%	*mM*	*mM*	%	*mM*	*mM*		*mM*	*mM*
1	24.5	1.9	0.11	98.1	5.42	0.1	0.01	5.57	99	2.80	3.13	89	1.25	0.31
2	11.5	2.3	0.13	97.5	5.64	0.3	0.02	5.67	102	2.84	3.08	92	1.46	0.41
3	17.0	2.8	0.14	93.9	4.54	3.2	0.15	4.94	98	3.02	3.18	95	1.21	0.35
4	10.4	1.7	0.11	98.0	6.23	0.4	0.03	6.50	98	3.70	4.09	91	1.51	0.37
5	12.4	2.5	0.05	97.1	1.99	0.5	0.01	1.95	105	1.82	1.79	102	1.29	0.43
6	11.3	2.1	0.16	96.7	7.25	1.1	0.08	7.34	102	2.05	2.53	81	0.71	0.08
Average	14.5	2.2	0.12	96.9	5.18	0.9	0.05	5.33	101	2.71	2.97	92	1.24	0.33

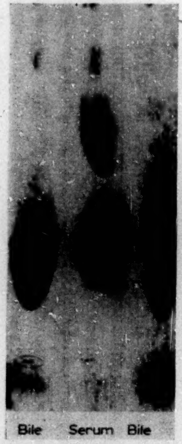

Fig. 1. Chromatography on silicic acid-impregnated filter paper with 20% methanol in chloroform elution of two lipid extracts of human bile compared to a lipid extract of human serum (center). Staining with the phosphomolybdic acid method reveals three spots with the serum extract, identified as lecithin, sphingomyelin, and lysolecithin in order of decreasing R_F. The bile extracts show a large lecithin spot, suggestion of a trace of sphingomyelin, and a lysolecithin spot in the sample on the left (Patient 3) and a suggestion of a lysolecithin spot in the sample on the right (Patient 4). The higher R_F of the lecithin spots of the bile extracts is due to the larger amount of material.

The concentrations of lecithin and free cholesterol in bile are more than double their respective concentrations in serum[5]. The secretion of such large amounts of lipid in bile suggests an important role for these substances in the digestive processes.

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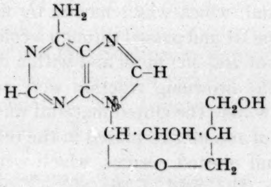
An improved method of isolation and determination of cordycepin

In 1951 CUNNINGHAM *et al.*[1] isolated cordycepin from liquid cultures of the mold *Cordyceps militaris* (Linn.) Link. The work of BENTLEY *et al.*[2] showed cordycepin to be an adenine nucleoside containing a 3-deoxypentose with a branched carbon chain.



Cordycepin

Apparently a large part of the metabolism of this organism is directed to the production of this substance since 25–100 mg of crystalline product can be isolated per liter of medium. This good yield of cordycepin offers the opportunity to study its biosynthesis, but a limitation to such studies is that the existing method of isolation requires some 20 days after harvesting.

This report describes an improved method of isolation of cordycepin, and in addition, it presents a colorimetric method for the quantitative estimation of cordycepose, the pentose moiety of the nucleoside.

Cordyceps militaris (Linn.) Link was obtained from Centraalbureau Voor Schimmelcultures, Baarn, Holland. The conditions for the production of cordycepin were essentially the same as those reported by CUNNINGHAM *et al.*[1], who grew the mold in culture flasks containing 400 ml of medium per flask. We have used 18-l pyrex bottles containing 13 l of medium each. The length of time required for the optimal production of the nucleoside was of the same order of magnitude reported for the smaller culture volumes[1].

All slant cultures were maintained on oatmeal agar prepared as follows: 30 g of quick-cooking rolled oats and 600 ml water were cooked for 30 to 45 min in a double boiler. This material was then strained through cheese cloth. 120 ml of this extract were autoclaved for 30 min with 280 ml water and 12 g agar. (10-ml portions of this were dispensed into test tubes, autoclaved, and slanted.) Cultures were transferred about every two weeks.

For the large-scale production of cordycepin a solution containing 65 g of enzymically prepared casein hydrolysate (Nutritional Biochemical Corporation) in 10 l water was autoclaved in an 18-l pyrex bottle at a pressure of 17 lb./in^2. for 2 h. A second solution containing 130 g glucose in 3 l water was autoclaved at the same time and added immediately afterward to the casein-hydrolysate solution. Slant cultures of *Cordyceps*, 2 to 4 weeks old, were shaken with 10 ml of a sterile 0.9 % saline solution. The resulting suspension of conidia was transferred to the 13 l of cooled medium, a total of 10 slants being used for the inoculum. The culture was allowed to grow at 23–25° without shaking. Usually in 2 days sub-surface growth was visible, while in 3 days small surface colonies began to appear. By 7–10 days a well-formed pad had developed at the surface of the medium. The surface culture in this form was allowed to grow for 20–30 days. At intervals aliquot portions were removed under sterile conditions and suitable dilutions made for absorbancy readings at 260 mμ.

When the density at 260 mμ reached a maximum, the mycelial pad was filtered off and the filtrate concentrated to approximately 0.1 its original volume in a flash evaporator (Precision Scientific, Catalog No. 65486) at a temperature not exceeding 50°. Storage of the concentrate for 12 to 24 h at 4° brought about the precipitation of a large quantity of material, which was removed by filtration. The filtrate was adjusted to pH 11 with 5 *N* NaOH and passed through a column 10 cm high × 71 cm^2 containing Dowex-1-chloride of 200–400 mesh and with a cross-linkage of 8. Most of the pigments produced by the browning reaction were adsorbed. Cordycepin was eluted by washing with 2–3 l water. The eluted material was concentrated in a rotary flash evaporator to a volume of 100 ml and placed in the refrigerator at 4° overnight. Cordycepin crystallized as dull matted needles, which were harvested by filtration and recrystallized from water. The yield of crystalline product from a 13-l batch of medium usually varied from 1.0 to 1.5 g. Physical and chemical data obtained on this product were in agreement with that reported previously[2].

Colorimetric determination of cordycepose

We observed that heating cordycepin with a slightly modified anthrone reagent[3] resulted in the production of a cherry-red color and this was investigated further as a means of quantitatively estimating cordycepin. The reaction was negative with adenine. The reagent was prepared fresh daily by dissolving 0.2 g anthrone (Eastman Kodak Co.) in 100 ml 90 % H_2SO_4 (150 ml water plus 900 ml conc. H_2SO_4). 5.0 ml of the anthrone reagent were added to 1.00-ml aliquot portions of solutions containing from 1–135 μg of cordycepin, mixed thoroughly and placed in an ice bath. A reagent blank was prepared using 1.00 ml water in place of the cordycepin. All tubes were removed from the ice bath and heated in a water bath at 100° for 10 min. The tubes were then cooled and compared with the blank in a Klett photometer utilizing a No. 52 filter. The chromophore produced was found to be quite stable for several hours and obeyed the Beer-Lambert law between 1 and 135 μg of cordycepin. In the routine determination of cordycepose a standard of cordycepin whose concentration was previously determined from the extinction coefficient at 260 mμ ($14.2 \cdot 10^3$) was run simultaneously with the blank and unknown. It was also found that the amount of color development in our procedure was the same whether the cordycepose was free or bound in the nucleoside. The absorption spectrum of the color produced in the anthrone determination of cordycepose is pictured in Fig. 1, along with those obtained with glucose, adenosine, and deoxyadenosine under identical conditions.

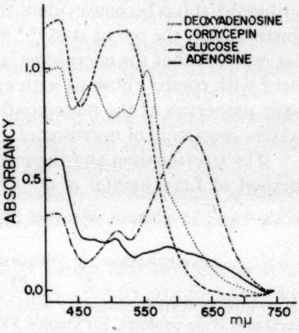


Fig. 1. Absorption spectra of pigments produced by the action of anthrone reagent on cordycepin, adenosine, deoxyadenosine, and glucose. 580 μg of cordycepin, adenosine, and deoxyadenosine were analyzed by the anthrone method as described in the text. In the case of glucose, only 100 μg was used. The absorption spectra recorded were read against a reagent blank in a Cary Recording Spectrophotometer.

The anthrone method has proved most useful to us in our studies, because we produce our material in the presence of glucose and occasionally in the presence of adenosine.

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Preliminary Notes

Incorporation of leucine into microsomal protein by a cell-free system of guinea-pig brain

As a result of work[1–3] carried out in several laboratories, including that of the authors[4,5], it has become evident that brain protein has rather high metabolic activity. Furthermore, the recent study[3,6] on [^{14}C]amino acid uptake into brain protein *in vivo* has revealed that the microsome is the most active site of protein synthesis, in agreement with results obtained with other tissues[7,8]. The present report describes several basic properties of the incorporation of [^{14}C]leucine into brain protein by a cell-free system consisting of microsomes and supernatant fluid of guinea-pig cerebral cortex.

The fractionation and the incubation were carried out according to the modified method of LITTLEFIELD *et al.*[9] (Table I). After the incubation of microsomes and

TABLE I

INCORPORATION OF [^{14}C]LEUCINE INTO THE BRAIN MICROSOMAL PROTEINS BY A CELL-FREE SYSTEM

The complete system contained washed or unwashed microsomes (3.0 mg protein), supernatant fraction (1.0 mg protein), 2.0 µmoles ATP, 0.25 µmole GTP, 20 µmoles creatine phosphate, 0.1 mg of creatine kinase and 0.5 µC [^{14}C]L-leucine (7.2 µC/µmole L-leucine). After incubation for 30 min at 37° in air, excess inactive L-leucine in 0.25 M sucrose was added to stop the reaction. Then microsomes and supernatant fraction were obtained by ultracentrifugation (105,000 × g, 90 min). From each fraction, protein was precipitated by addition of trichloroacetic acid, and purified by a routine method, eliminating non-protein bound amino acids, RNA and lipids. Radioactivities of dried materials were measured in a gas-flow counter and specific activities were expressed as counts/min/mg protein.

	Microsome protein (counts/min/mg)	*Supernatant protein (counts/min/mg)*
A. Complete system (unwashed microsome)	1110	213
supernatant fraction omitted	844	78
Complete system (washed microsome)	1030	154
supernatant fraction omitted	266	17
B. Complete system (washed microsome)	498	83
supernatant fraction omitted	66	18
ATP omitted	19	7
GTP omitted	266	33
Creatine phosphate-creatine kinase omitted	19	12
C. Complete system (washed microsome)	486	110
RNase added (100 µg)	21	12
RNase added (0.5 µg)	46	17

Abbreviations: ATP, adenosine triphosphate; GTP, guanosine triphosphate; RNase, ribonuclease; RNA, ribonucleic acid.

supernatant fluid with uniformly labeled [^{14}C]L-leucine, the cell fractions were separated by ultracentrifugation. The protein of each fraction was then subjected to purification and the specific activity was determined as described previously[5]. The results obtained by treatments of this purified protein with 2,4-dinitrofluorobenzene and with performic acid respectively suggested that the labeled leucine was incorporated into the peptide chain of protein molecules.

In a complete system, including ATP, GTP and ATP-generating system, microsomal protein was labeled rapidly and reached the maximal specific activity after about 15 or 30 min incubation. Release of [^{14}C]labeled protein from microsomes into the soluble fraction was always observed as shown in Table I. The requirement for supernatant was demonstrated by the use of once-washed microsomes. It was noted that the pH-5 enzymes fraction was not as effective for the incorporation as the supernatant fraction. As shown in Table I (B), little incorporation occurred in the absence of ATP or an ATP-generating system. It was further shown that GTP is required for the maximal incorporation. Similar results were obtained in the experiment in which the pH-5 enzymes fraction was used instead of supernatant fluid.

RNase, even at a concentration of 0.5 μg/ml, inhibited markedly the incorporation of [^{14}C]leucine into both protein fractions examined. An amino acid mixture (20 amino acids except L-leucine) did not stimulate the incorporation. Amobarbital (10^{-3}–10^{-5} M) was not inhibitory in this system, although the inhibitory action of barbiturate on the incorporation of [^{14}C]amino acid into the protein of brain slices has been reported[10].

These results seem to suggest that amino acid-activating enzymes, soluble RNA and the microsome system are important for the synthesis of brain protein as in other tissues. It is of interest that the Nissl granules in the nerve cell are identified as microsomes by ultracentrifugal and electron microscopical examination[11].

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Formation of deoxyguanosine 5′-phosphate from guanosine 5′-phosphate with enzymes from chick embryos

Incorporation experiments with labeled pyrimidine ribonucleosides originally demonstrated that deoxyribosyl compounds could be formed through a reduction of ribosyl compounds (cf. ref. 1). More recently, the formation of deoxy-UMP and deoxy-CMP from the corresponding ribonucleotides was demonstrated with soluble enzymes from chick embryo and *Escherichia coli*, respectively[2,3]. With the cytosine derivatives, requirements for ATP, Mg^{++} and TPNH were found; the dependence of the reaction on TPNH was shown by treating the bacterial extract with Dowex-2-Cl$^-$. Furthermore, it was demonstrated that deoxy-CDP is the first product of the reaction, and therefore it was considered likely that the actual reduction step occurred at the diphosphate level.

For purine nucleotides, the earlier evidence from experiments *in vivo* was less conclusive for a direct formation of deoxyribosyl purines from ribosyl purines[4]. However, results have now been obtained with extracts of 5-day-old chick embryos (25,000 × *g* supernatant fraction) which demonstrate a direct transformation of guanosine ribonucleotides to deoxyribonucleotides.

Table I describes the conditions of incubation for the optimal formation of deoxyguanosine phosphates from [8-^{14}C]GMP and indicates that the reaction was dependent on ATP and Mg^{++}. In each experiment the reaction was stopped by boiling, 0.1 *M* KOH (0.005 ml), 0.5 *M* MgCl$_2$ (0.01 ml) and 0.1 mg of crude *Crotalus adamenteus* venom were then added, and the mixture was incubated at 37° for 60 min to dephosphorylate all nucleotides. After heat inactivation, carrier deoxyguanosine was added, and the total supernatant fraction after centrifugation was chromatographed on paper (Whatman 3 MM) using a borate solvent[5]. This procedure separated deoxyguanosine (R_F = 0.49) completely from guanine (R_F = 0.34), guanosine (R_F = 0.18) and xanthine (R_F = 0.26). From the total radioactivity in the deoxyguanosine spot the amount of deoxyguanosine compounds formed could be calculated.

TABLE I

REQUIREMENTS FOR THE FORMATION OF DEOXY-GMP

The complete system contained: 27 mμmoles [8-^{14}C]GMP (3.0·10^6 counts/min/μmole), 0.25 μmole ATP, 1.5 μmoles MgCl$_2$, 3.3 μmoles Tris, pH 8.0, and 1.3 mg protein. Volume, 0.15 ml. Incubation for 30 min at 37°. For further explanation see text.

Omission or addition	*mμmole deoxyguanosine found*
None	0.189
None*	0.029
None**	0.008
Minus ATP	0.015
Minus MgCl$_2$	0.076
Plus 2 μmoles EDTA	0.008

* The treatment with snake venom to dephosphorylate deoxyguanosine phosphates was omitted.
** The reaction mixture was boiled prior to incubation.

Abbreviations: UMP, uridine 5′-monophosphate; CMP and CDP, cytidine 5′-mono and di-phosphate; GMP and GDP, guanosine 5′-mono- and di-phosphate; ATP, adenosine 5′-triphosphate; TPNH, reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane–HCl buffer; EDTA, ethylenediaminetetracetic acid, sodium salt.

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For further identification the deoxyguanosine isolated by paper chromatography was rechromatographed on Dowex-50-NH_4^+. Of the total radioactivity added to the column 88 % was recovered in the deoxyguanosine peak and, furthermore, the ratio of ultraviolet absorption to radioactivity remained constant in each of the effluent fractions of the peak.

Evidence for maintainance of the glycosyl linkage during deoxy-GMP formation is presented in Table II. In this experiment ^{14}C-randomly labeled GMP was used as the substrate for the formation of deoxyguanosine derivatives. The specific activities of the deoxyguanosine and of the guanine portion obtained after acid degradation of deoxyguanosine were determined. The ratio of the specific activity of the guanine to that of the sugar was found to be very similar in both GMP and deoxyguanosine.

TABLE II

DEGRADATION OF DEOXYGUANOSINE FORMED FROM RANDOMLY LABELED GMP

Randomly labeled [^{14}C]GMP (0.16 µmole, 1.6·10^6 counts/min/µmole) was incubated at 37° for 45 min with 3 µmoles ATP, 12 µmoles $MgCl_2$, 27 µmoles Tris, pH 8.0, and 10.4 mg protein in a final volume of 0.98 ml. The reaction was stopped by boiling. Carrier deoxyguanosine (2 µmoles) was added and the nucleoside was isolated by chromatography after treatment of the reaction mixture with snake venom.

Compound	*Specific activity (counts/min/µmole)*			
	Nucleoside	*Guanine*	*Sugar*	*Guanine/sugar*
Randomly labeled GMP*	4,330	1,750	2,580	0.68
Deoxyguanosine formed	825	317	508	0.62

* Determined after addition of carrier.

A close similarity is apparent between the reactions leading to the formation of deoxyribosyl phosphates from both GMP and CMP. Both types of reactions show sharp optima in their ATP and Mg^{++} requirements and are inhibited by the addition of an ATP-regenerating system, such as creatine phosphate and kinase. It appears probable that deoxy-GDP is the first deoxyribosyl compound formed from GMP, although direct evidence on this point has not yet been obtained.

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A pepsin preparation without transpeptidation activity

Pepsin was shown to possess transpeptidation activity towards synthetic substrates[1], in addition to its well-known hydrolytic activity. In this communication we describe the preparation of a pepsin, to be denoted as "pH-3 pepsin", which does not exhibit transpeptidation activity and yet preserves its hydrolytic activity.

The "pH-3 pepsin" was prepared as follows: 1 g pepsinogen (Nutritional Biochemical Corp., Cleveland, Ohio; lots No. 7546 or 7809 were used without further purification) was dissolved in 50 ml sodium phosphate buffer, pH 6.8, I 0.02. The pH of this solution was adjusted to 3.0 with 1 *N* HCl. After 1 h sat. aq. $MgSO_4$ was added to bring the salt concentration to 0.6 satn. This was followed by centrifugation at 12,000 × *g* for 60 min. The precipitate formed was dissolved in a small volume of 0.1 *M* sodium acetate, pH 4.7, and then reprecipitated with $MgSO_4$ at 0.6 satn. This final precipitate was dissolved in 10 ml 0.1 *M* sodium acetate buffer, pH 4.7, and stored in the cold. The yield was about 100 mg.

As a control, a pepsin preparation obtained by activation of pepsinogen at pH 2[2] was used. This "pH-2 pepsin" was isolated from the activation mixture in the same manner as "pH-3 pepsin". From 1 g pepsinogen, 250 mg of "pH-2 pepsin" were obtained.

The enzymic properties of "pH-3 pepsin" and of "pH-2 pepsin", respectively, are presented in Table I. It is apparent that the two preparations have similar activities towards hemoglobin[3]. They differ markedly, however, in their behaviour towards synthetic substrates. When the "pH-3 pepsin" was incubated with substrates, such as N-carbobenzoxy-L-phenylalanyl-L-tyrosine or N-carbobenzoxy-L-tyrosyl-L-tyrosine, known to undergo with pepsin both hydrolysis and transpeptidation[1], only hydrolysis occurred. On the other hand, with "pH-2 pepsin", as with crystalline pepsin, the same substrates gave both hydrolysis and transpeptidation.

TABLE I

	Specific activity			
	*PU Hb/mg N**		*PU ZTyrPhe/mg N***	*Transpeptidation****
	at pH 1.7	*at pH 3.2*	*at pH 4.7*	
"pH-2 pepsin"	0.14	0.033	0.29	+
"pH-3 pepsin"	0.13	0.034	0.80	—
"pH-3 pepsin" treated at pH 2	0.13	0.034	0.38	+
crystalline pepsin	0.16	0.034	0.40	+

* Assayed at pH 1.7 according to the method of ANSON[3]. For assays at pH 3.2, a 2 % solution of acid-denaturated hemoglobin in citrate-phosphate buffer[5] was used, the other steps of the procedure being the same as at pH 1.7.

** PU ZTyrPhe = μmole phenylalanine released/h upon incubation of enzyme in a solution containing 2.0 mg/ml N-carbobenzoxy-L-tyrosyl-L-phenylalanine in 0.1 *M* sodium acetate buffer, pH 4.7, at 37°. Phenylalanine was assayed by the ninhydrin method of COCKING AND YEMM[6].

*** Incubation mixtures at pH 4.7, containing N-carbobenzoxy-L-phenylalanyl-L-tyrosine or N-carbobenzoxy-L-tyrosyl-L-tyrosine, were analysed by paper chromatography and paper electrophoresis as already described[1]. Transpeptidation was observed either by the formation of higher peptides or by the appearance of L-tyrosyltyrosine.

+ Transpeptidation found.

— No transpeptidation.

Transpeptidation in all the above cases was tested by the formation of corresponding tripeptides such as carbobenzoxy-L-phenylalanyl-L-tyrosyl-L-tyrosine and that of L-tyrosyl-L-tyrosine, the latter being a product of the hydrolysis of tripeptides.

An additional difference was observed when the action of the various preparations was tested on synthetic substrates which with crystalline pepsin have been shown to undergo hydrolysis only[4]. These substrates include the N-acylated dipeptides with C-terminal phenylalanine. The rate of hydrolysis at pH 4.7 of one of such substrates, N-carbobenzoxy-L-tyrosyl-L-phenylalanine, by "pH-3 pepsin" was approximately two times higher than that by crystalline pepsin (Table I).

The "pH-3 pepsin" could readily be converted to "pH-2 pepsin" by lowering the pH to 2 with HCl.

The results reported above seem to indicate that either (a) "pH-3 pepsin" is an intermediate formed during the activation process of pepsinogen, or (b) "pH-3 pepsin" contains a factor inhibiting transpeptidation which can be removed on acidification to pH 2.

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Isolation of "kepalin B" from cerebral lipids

In 1949 Brante[1] gave evidence for the existence of a hitherto unknown phospholipid in human and animal nerve tissue. It was acid and alkali stable, contained amino nitrogen but no choline, which is the reason Brante proposed the name "kephalin B" for the new lipid. Later Dawson[2], Edgar[3] and Weiss[4] have reported similar findings. Recently Carter *et al.*[5] have shown that a phospholipid from egg yolk, with similar behaviour to the compound indicated by Brante, was a glycerol ether phospholipid consisting mainly of a phosphorylethanolamine derivative of batyl alcohol.

In the present paper we give a preliminary report on the isolation of a similar substance from calf and human brain. Homogenized brain material was extracted with chloroform–methanol (2:1, v/v). Water-soluble contaminants were removed by partition against 1 % saline. The lipid extract was subjected to chromatography on silicic acid. The column was prepared from reactivated silicic acid (Baker A.R.) passing 300 mesh. The different groups of lipids were eluted with a solvent mixture of chloroform and stepwise increasing amounts of methanol. Chloroform–methanol

(4:1, v/v) eluted cerebrosides, sulfatides and ninhydrin-positive lipids. The lipids were identified by quantitative analyses for nitrogen, hexose, phosphorus and ester-bound fatty acids and by qualitative paper chromatography of the intact lipids. The ninhydrin-positive fractions were subjected to the mild alkaline hydrolysis procedure of SCHMIDT *et al.*[6] (1 *N* KOH at 37° for 24 h). After neutralisation the lipids were extracted with chloroform and rechromatographed. The ninhydrin-positive lipids were found by some overlapping after the sulfatides. A complete separation was achieved by chromatography on magnesium silicate (Florisil, 60–100 mesh). The main part of the sulfatides were eluted with chloroform–methanol (2:1, v/v). After the addition of 2% water to the eluting solvent, remaining sulfatides and the ninhydrin-positive material were eluted in two distinct peaks.

The percentage yields of the new phospholipid depended upon the material used. In general it was greater in lipid extracts from young subjects. The yield from 10 g of calf-brain lipids was 225–250 mg, from child-brain lipids 200–225 mg and from adult-brain lipids 100–150 mg. The new lipid has also been isolated from human liver, kidney and spleen.

Analysis of the phospholipid from calf-brain lipids showed the following figures (for comparison the figures found by CARTER *et al.*[5] are also given):

CARTER C, 57.37; H, 10.08; N, 3.24; P, 6.24; N:P, 1.15; LCB–N, 0.11

Present C, 58.02; H, 9.82; N, 2.81; P, 6.29; N:P, 0.99; LCB–N, 0

After acid hydrolysis (2 *N* HCl for 2 h) only ethanolamine and phosphatidic acid were identified by paper chromatography. There was no trace of serine. The amount of inorganic P was negligible. Acetolysis[7] of the lipid gave large amounts of inorganic P.

In an attempt to answer the question whether the compound occurs in the tissues in an unesterified or esterified form, several unsuccessful attempts were made to isolate the new lipid by non-hydrolytic procedures. But it was found that the alkali-resistant lipid was uniformly distributed in the ninhydrin-positive fractions obtained by silicic acid chromatography. However, when the new lipid was added to a unhydrolysed lipid extract it was eluted after the other ninhydrin-positive lipids. This supports the hypothesis that the new lipid occurs in esterified form in nature and may be regarded as a plasmalogen with a saturated aldehydogenic residue.

In the alkali-treated lipid extracts we have also found small amounts of a choline-containing lipid analogues to the compound described. In silicic acid chromatography it is eluted after the sphingomyelins.

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Origin of the ribonucleic acid associated with the soluble antigens of tobacco mosaic virus

Most investigators who have isolated proteins serologically related to tobacco mosaic virus from infected plant extracts (soluble antigen, X protein, etc.) did not find them associated with RNA[1,2]. Nevertheless, Bawden and Pirie[3] consider that they are virus-like ribonucleoprotein, since their preparations always contain 0.1–0.2 % RNA phosphorus. This discovery of Bawden and Pirie might lead to a better understanding of the mechanism of TMV synthesis *in vivo* if these proteins, which take up labelled amino acids at a very high rate, are really virus precursors[4].

We have tried to confirm the existence of bonds between soluble antigens, found in plant extracts and RNA, and also to determine the origin of the latter.

The soluble antigens were precipitated by a specific antiserum after preliminary removal of cellular debris and virus particles as has been described previously[4]. It seemed unlikely that this isolation method would break the bonds between RNA and soluble antigens postulated by Bawden and Pirie. In fact, we have found that the virus antigen precipitated by this method contains approximately 1 % RNA. ^{32}P-labelling experiments carried out during the period of virus multiplication show that, after 2-h incubation of the infected leaves with labelled phosphate, the specific radioactivity of soluble antigen RNA is three times higher than that of TMV RNA. This fact alone excludes the possibilities that soluble antigen RNA belongs either to contaminating TMV particles or even to small fragments of TMV, disrupted during homogenization. On the other hand, calculations based upon the total radioactivity incorporated into TMV RNA during the ^{32}P-incubation period show that the mean specific radioactivity of soluble antigen RNA for this period is ten times lower that one would expect for a TMV RNA precursor. The bulk of the soluble antigen RNA is therefore neither a precursor nor a degradation product of infectious virus.

A comparison between the base ratios of RNA derived from soluble antigen, TMV and tobacco plant (Table I) indicates that the soluble antigen RNA is very likely tobacco-plant RNA.

TABLE I

PURINE AND PYRIMIDINE CONTENTS OF RIBONUCLEIC ACIDS FROM TMV, SOLUBLE ANTIGEN AND TOBACCO-LEAVES EXTRACTS

The values are the means of 6–8 determinations.

RNA moles/100 moles	*TMV*	*Soluble antigen*	*Tobacco leaves, pellet**	*Tobacco leaves, supernatant**
Guanine	25.1	31.1	29.8	31.0
Adenine	28.9	28.6	25.5	24.8
Cytosine	19.1	20.1	24.1	24.3
Uracil	26.9	20.2	20.6	19.9

* After centrifugation at 28,000 rev./min for 20 min in Spinco rotor 30.

It remained to be seen whether the presence of plant RNA in soluble antigen precipitates is due to bonds existing within the cells between antigen and plant RNA

Abbreviations: RNA, ribonucleic acid; TMV, tobacco mosaic virus.

or plant ribonucleoprotein or whether it is due to an artefact arising during the extraction or the precipitation process.

An unlabelled infected-plant extract from which TMV particles have been removed by centrifugation was mixed with an uninfected ^{32}P-labelled plant extract. The mixed extracts were treated with anti-TMV serum. The specific activity of RNA nucleotide phosphorus in the precipitate amounted to 0.5 % of that of RNA nucleotide P of labelled healthy plant. This shows that no appreciable contamination of soluble antigen by plant RNA occurs after mixing the extracts or during precipitation.

In a second experiment, equal weights of fragments of unlabelled infected leaves and ^{32}P-labelled healthy leaves were thoroughly mixed and then homogenized together. In this way, the content of labelled and unlabelled cells were mixed immediately after cell disruption. Under these conditions, the specific radioactivity of the RNA nucleotide phosphorus obtained from immunological precipitates of soluble antigen reached approximately half of the specific activity of healthy-plant nucleotide phosphorus.

This is exactly what we must expect if the bulk of the RNA associated with the soluble antigen derives from the formation of complexes between antigen and plant RNA immediately after cell disruption. This plant RNA is in fact a mixture of equal parts of labelled plant RNA from the healthy leaves and non-labelled plant RNA from the infected leaves.

The presence of large amounts of RNA in soluble antigen prepared under very mild conditions does therefore appear of very little significance.

It is, however, possible that a small fraction of soluble antigen RNA is in reality TMV RNA already embedded within the first few protein turns of virus particles in formation. This RNA fraction would obviously have a high turnover rate and in short-time labelling experiments, the specific radioactivity of the antigen RNA would have to be much higher than that of plant RNA. In fact, this is so in such experiments where, notwithstanding the presence of contaminating plant RNA, the specific radioactivity of soluble antigen RNA is about 10-fold higher than that of plant RNA remaining after immunological precipitation of the antigen. Nevertheless, calculations based upon the total ^{32}P radioactivity incorporated in virus particles during the experiments show that only 10 % antigen RNA needs to be virus RNA to account for the high specific radioactivity of the antigen RNA.

Consequently, if soluble antigen precipitates contain a virus-specific RNA, this could not be shown by base analysis because of the presence of contaminating plant RNA in large amounts.

A study of antigen-bound virus-specific RNA which might be present would necessitate the preliminary separation of the contaminating plant RNA.

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Changes in protein-bound acid-amide ratios during culture of Walker carcinosarcoma 256 cells *in vitro*

It is well known that mammalian cells *in vitro* exhibit proliferation cycles, *i.e.*, lag, logarithmic, and stationary phases, which are similar to those of microbial cultures. Recently[1–7] some correlations of phases of these cycles with changes in cellular content of nucleic acids, lipids, and protein have been described for established strains of mammalian cells *in vitro*. In the present study changes in composition, as well as content, of the protein in Walker carcinosarcoma 256 cells were measured during the transition from an *in vivo* environment to proliferation *in vitro*. In the course of this study, as the cells progressed through the proliferation cycle, alterations were observed in the amount of protein-bound aspartic and glutamic acids relative to their respective amides, asparagine and glutamine.

Cell suspensions of freshly excised 13-day Walker tumors were cultured as monolayers in T-60 flasks. The medium was that of NEUMAN AND McCOY[8], subsequently modified[9], and the concentrations (mM), of 4 amino acids were changed as follows: arginine, 0.2 to 0.4; histidine, 0.1 to 0.2; lysine, 0.2 to 0.6; hydroxyproline, 0.15 to 0.015. The cultures were gassed with 5 % CO_2 in air.

The protein from an aliquot of the initial inoculum was extracted[10], hydrolyzed in 6 N HCl, and analyzed on Amberlite CG-120 by the procedure of MOORE *et al.*[11]; this was considered to be representative of tumor-cell protein *in vivo* at time of excision. After an initial 5-h incubation period to allow cells to adhere to the glass surface, the medium and floating cells were replaced with fresh medium and a portion of the cultures was removed for cell counts and protein analysis. The counting and protein analysis of sticking cells were repeated at various incubation times thereafter.

Since all the protein samples were hydrolyzed in acid, the micromoles of aspartic and glutamic acids measured from the ion-exchange column actually represented the sum of these acids plus asparagine and glutamine. The contributions of the amides to these totals were calculated by assuming that a portion of the ammonia content of the hydrolysates arose from a 5 % and 10 % destruction of threonine and serine, respectively, in the manner reported by REES[12] and by HIRS *et al.*[13]. The corrected

TABLE I

CHANGES IN PROTEIN-BOUND CONTENT OF ASPARTIC AND GLUTAMIC ACIDS AND THEIR AMIDES RELATIVE TO PHASES OF THE PROLIFERATION CYCLE OF WALKER CARCINOSARCOMA 256 CELLS *in vitro**

Incubation time (h)	0**	5	17	36	44	52	60	72
Growth (Cells/Flask × 10^{-6})	4.0	3.9	2.8	4.0	5.4	6.7	8.3	8.8
Protein constituent (μmoles/10^8 cells)								
Aspartic acid	10.67	8.49	10.34	20.70	21.89	20.84	17.55	21.57
Glutamic acid	13.84	10.96	13.15	26.42	28.17	24.91	24.99	26.89
Amide NH_3	11.47	9.23	11.46	17.65	17.57	16.99	17.39	20.89
Ratio: acids/amides	1.14	1.11	1.05	1.67	1.85	1.69	1.45	1.32
Mole % amides in total moles***	9.28	9.68	9.93	8.07	7.58	7.78	8.06	9.23

* For culture conditions see text.

** Represents the cell suspension prepared from freshly excised tumors and used for the initial inoculum of $4 \cdot 10^6$ cells per flask; 5–17 h, lag phase; 17–60 h, proliferation phase; 60–72 h, retardation phase.

*** Calculated from the complete protein amino acid analyses.

ammonia values represented, then, an approximation of the amide content of the original protein (assuming the contribution from destruction of other amino acids to be constant). The results of these measurements are shown in Table I. From the acid/amide ratios, it can be seen that the protein was relatively enriched in amide content during the lag phase, but it contained a considerably higher proportion of free acids during the proliferation phase. As the cells began to enter the stationary phase (60–72 h) the amide content in the protein again rose. These changes in amide content are expressed in Table I also as percent of total moles of amino acids recovered in the amide amino acids.

From these results it could appear that the proliferation process required a greater proportion of one or both amides for functions other than protein synthesis than was required in the lag or retardation phases. This explanation of the above results is more likely, perhaps, than two other possibilities, *i.e.*, the proteins with higher free acid content were more active enzymically or that the amide functions were removed from intact proteins.

Preliminary studies with Jensen sarcoma cultures indicate a similar phenomenum, and analogous studies are in progress with tumors proliferating *in vivo*. No previous evidence for correlation of changes of protein amino acid composition with phases of proliferation cycles of mammalian cells has come to the attention of the authors.

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NEW EVIDENCE FOR THE ROLE OF ACETYLCHOLINE IN CONDUCTION

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SUMMARY

Ranvier nodes of single frog sciatic nerve fibers and desheathed bundles of frog tibialis nerve have been exposed to several compounds known to react with the acetylcholine system. The effects on electrical activity and on membrane properties have been studied. Eserine, a strong inhibitor of acetylcholinesterase, reversibly blocks conduction at Ranvier nodes in concentrations of 200–300 μg/ml within 30 sec; on intact frog sciatic nerve 20–30 min are required to obtain the same result with eserine at 3–5 mg/ml. 1.5 to 15 μg/ml eserine increases the spike amplitude and prolongs its descending phase. Lipid-soluble dimethyl aminoethyl acetate, when applied to Ranvier nodes at 1 mg/ml, increases spike height and prolongs the descending phase. When applied in combination with eserine (1.5 μg/ml) it blocks conduction reversibly. A lipid-soluble quaternary ammonium compound, pyridine-aldoxime dodeciodide blocks conduction of Ranvier nodes; at $10^{-6}\,M$ the effect is reversible, while at $5 \cdot 10^{-6}\,M$ it becomes irreversible. At lower concentrations ($10^{-7}\,M$) pyridine-aldoxime dodeciodide increases the amplitude and duration of the spike. Pyridine-aldoxime dodeciodide and other lipid-soluble quaternary ammonium compounds, applied to desheathed frog tibialis nerve at concentrations which do not affect the resting potential, increase the permeability to sodium, that to potassium appears slightly decreased. The mechanism of the action of these compounds is discussed. The effects offer new evidence for the role of acetylcholine in the conduction of nerve impulses.

INTRODUCTION

It is widely accepted today that electrical activity is associated with characteristic movements of sodium (Na) and potassium (K) ions. When an impulse is conducted along a nerve fiber Na moves inwards while K moves outwards; during activity there is a change in the conductance of these two ion species. NACHMANSOHN[1,2] has proposed that the acetylcholine system is essential for these conductance changes; during the last two decades he and his colleagues have accumulated a large amount of evidence in favor of this view.

One of the earliest indications for the role of acetylcholine in the generation of bioelectric currents in general was the finding of the strikingly high concentration of acetylcholinesterase in electric organs and the depolarizing action of the ester in this tissue. In recent years depolarizing actions were observed with microelectrodes on myoneural and synaptic junctions; but the absence of a similar action on the axon was one of the main objections to NACHMANSOHN's concept of a similar function of the ester in conducting membranes in general. NACHMANSOHN attributed this lack of response to the existence of structural barriers impervious to lipid-insoluble quaternary ammonium ions such as acetylcholine. He and his colleagues have offered experimental evidence for this explanation in a variety of ways. One line of observation was the demonstration that lipid soluble inhibitors of acetylcholinesterase, such as eserine, block conduction, whereas lipid insoluble quaternary analogues such as prostigmine are completely inactive.

This argument was challenged by DEL CASTILLO AND KATZ[3] on the basis that the high concentration of eserine required was much higher than the concentrations effective at junctions. This objection may be answered in two ways: (a) to study the effects on preparations where there is reason to believe that the structural barrier is relatively weak, and (b) to change the structure of specific compounds in such a way that they penetrate the barrier more readily.

A preparation which appeared suitable is the isolated frog sciatic nerve fiber; electron microscope studies have revealed that at the nodes of Ranvier the conducting membrane is covered by a very thin structure. The second line of approach became possible by the availability of lipid-soluble quaternary ammonium ions. Experiments along both lines have been carried out and are reported in this paper.

METHODS

Resting and action potentials were recorded with the technique described by STÄMPFLI[4, 5]. For measuring the resting potential desheathed frog tibialis nerves were used, while for the experiments recording the action potentials the single nerve fiber technique was employed.

Pyridine-2-aldoxime dodeciodide (PAD) and acetoxyethyldimethyl dodecyl ammonium iodide (noracetylcholine 12) were used as lipid soluble quaternary ammonium ions. The compounds were provided by Dr. S. GINSBURG.

Procedure

The same preparation was tested several times, alternately, with the control solution and with the solution containing the compound being studied. The mean values of the changes in resting potential in the absence of the compound were compared to those in its presence. The ion composition of the control and test solution was prepared in such a way that they differed only in either K or Na content. In the observations on the action potential the ion composition of the solution was not modified; the compound tested was added at varying concentration to Ringer's solution. In this type of experiment the Ringer's solution was also applied before the addition of the compound, then in its presence, and finally again without it.

RESULTS

Eserine

The dissociation constant of eserine is $8 \cdot 10^{-9}$ at 20° (see ref. 6). At pH 5.9, 99 % of the molecules are, therefore, in the cationic form; at pH 10, 0.6 %. The cationic (protonated) form has been shown *in vitro*, to be more effective as an inhibitor of acetylcholinesterase than the conjugate base[7]. On the intact nerve the neutral molecule is more effective. It has been found that the effectiveness of eserine increases with increasing levels of pH[8]; a roughly linear relationship exists between the concentration of the conjugate base in solution and the percentage attenuation of the action potential. The results obtained *in vitro* and on the intact cell are readily compatible, if one keeps in mind that the neutral molecule may penetrate and therefore act on interior sites[9–13], whereas the cationic species does not enter the cell. Once the molecules are inside, the pH level at the site of action will regulate the equilibrium between the two forms.

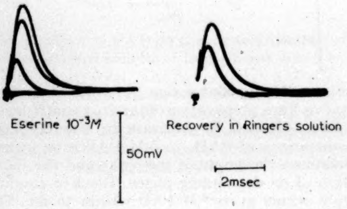

Fig. 1. Effect of eserine on the mononodal action potential of a single nerve fibre preparation of the frog sciatic nerve (*Rana pipiens*). At $10^{-3}M$ concentration (300 μg/ml) eserine blocks conduction in 30 sec. After the return to Ringer's solution conduction was restored within 40 sec. The spike reached its original height after 10 min. pH 7, 7. Temperature, 23°.

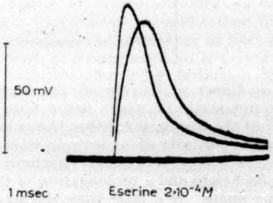

Fig. 2. Effect of eserine on the mononodal action potential of a single nerve fibre preparation of the frog sciatic nerve (*Rana pipiens*). Eserine at a concentration of $2 \cdot 10^{-4}$ *M* (60 μg/ml) reduces the spike height but increases the duration. Block of conduction occurs only after 15–20 min. The decrease of the action potential is reversible. pH 7,7. Temperature, 23°.

In the experiments to be reported here, the pH of the Ringer's solution was kept between 7.6 and 7.9, so that a considerable fraction of the compound was in the neutral form, although still less than 50 %. At a concentration of 10^{-3} *M* (equivalent to 300 μg/ml) conduction was blocked within 25 sec. On return to Ringer's solution conduction reappeared within 30 sec, although more than 10 min were required until the action potential returned to its original amplitude (Fig. 1). At lower concentrations, $2 \cdot 10^{-4}$ *M*, block of conduction occurs, if at all, only after 15–20 min, but the action potential is reversibly decreased (Fig. 2).

A qualitatively different effect was observed with still lower concentrations varying from $5 \cdot 10^{-6}$ *M* to $5 \cdot 10^{-5}$ *M* (equivalent to 1.5–15 μg/ml). On exposure to these low concentrations, first an increased spike height and a prolongation of the descending phase is observed. At a later phase the amplitude may decrease (Fig. 3). When the descending phase (repolarization) is prolonged, the latency of the subsequent action potential is increased. All the effects are reversible. In all cases, there is a definite threshold increase of the stimulus strength.

At more alkaline pH, 8.5, eserine blocks even at a concentration of 10^{-5} *M* (about 4 μg/ml).

Pyridine-2-aldoxime dodeciodide (*PAD*)

PAD is a lipid-soluble quaternary ammonium ion, and due to its solubility in lipids it penetrates into the interior of the axon and depolarizes the conducting membrane[14, 15]. When applied to Ranvier nodes of a single fiber preparation from frog sciatic nerve, PAD at a concentration of $5 \cdot 10^{-6}$ *M*, blocks conduction irreversibly within seconds. In 10^{-6} *M* concentration PAD produces within the first 30 sec, an increase of threshold, and a prolongation of the descending phase. After 15 additional seconds conduction is blocked. On return to Ringer's solution the original action potential is restored within 30 sec. At concentrations of 10^{-7} *M* PAD prolongs the descending phase, whereas the amplitude of the action potential remains either unchanged or is slightly increased. All these changes take place within seconds (Fig. 4). On return to Ringer's solution the original characteristics of the action potential are again restored within seconds.

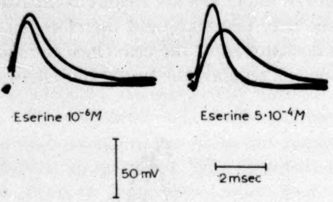

Fig. 3. Effect of eserine on the mononodal action potential of a single nerve fibre preparation of the frog sciatic nerve (*Rana pipiens*). Eserine in concentrations varying from $5 \cdot 10^{-6}$ *M* to $5 \cdot 10^{-5}$ *M* (1,5–15 μg/ml) effects an increased spike height and a prolongation of the descending phase. At a later time the amplitude decreases but the prolongation remains or becomes even larger. Eserine at a concentration of $5 \cdot 10^{-4}$ *M* reduces the spike height but increases the duration. pH 7,8. Temperature, 24°.

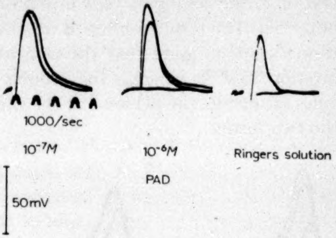

Fig. 4. Mononodal action potential of a single nerve fibre preparation of the frog sciatic nerve (*Rana pipiens*) on exposure to different concentrations of PAD. 10^{-7} *M* PAD (0.04 μg/ml) increases the height of the spike and the duration of its descending phase. Block of conduction occurs at 10^{-6} *M* PAD within 30 sec. The action potential reappears within 40 sec after return of the fibre to Ringer's solution. pH 7,8. Temperature, 24°.

If these lipid-soluble analogues affect specifically the acetylcholine system and depolarize by an activation of the receptor, then a receptor inhibitor should have a protecting effect by competing for the receptor. Evidence in this direction had been offered in the experiments in which competition was demonstrated between eserine and PAD[15, 16].

Permeability to potassium

A particularly pertinent question is that of a selective change in ion permeability, since during activity Na conductance specifically increases several hundredfold, while the increase in K conductance follows later and is relatively small. In order to test the action of lipid-soluble quaternary ammonium ions upon the permeability to K and Na, the rate and the degree of the changes of the resting potentials, evoked by variations of the K and Na concentrations in the bathing fluid, were measured. An increase of the K concentration from 2.5 m*M* to 20 m*M* produces a depolarization which rapidly reaches a plateau. When the nerve preparation is returned to Ringer's solution, the potential difference regains its initial value. If PAD is added to the Ringer's solution at a concentration which does not affect the resting potential, *i.e.*, does not produce a measurable change, the rate and the degree of depolarization are slightly, but significantly decreased (Fig. 5); the rate of repolarization is also slightly lower than in the control. In the absence of PAD the depolarization produced by 20 mM K reaches a stable level within 30 sec, whereas in its presence the level is reached only after 2 min. After the removal of PAD and after prolonged washing with Ringer's solution no marked change of K depolarization takes place, indicating poor reversibility. For quite some time the membrane remains less sensitive in its response to K than before the exposure (Table I).

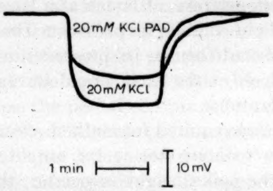

Fig. 5. Effect of PAD on the depolarization of the desheathed bundle of frog tibialis nerve by K^+ (*Rana pipiens*). The figure shows a depolarization produced by 20 mM K^+ and the repolarization in a 2.5 mM K Ringer's solution. On the addition of PAD (10^{-5} M) the depolarization by 20 mM K^+ is smaller and returns at a slower rate. Temperature, 24°.

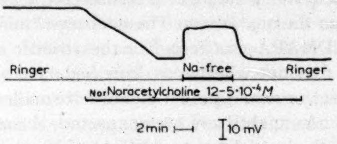

Fig. 6. Effect of noracetylcholine 12 on the membrane potential of the desheathed bundle of frog tibialis nerve in absence of Na^+. Noracetylcholine, at a concentration of $5 \cdot 10^{-4}$ M in Ringer's solution, causes depolarization. Replacement of Na by choline chloride in the Ringer's solution (Na^+ free) results in a repolarization. On the addition of Na^+ a rapid depolarization takes place again. This effect may be repeated several times. Temperature, 24°.

TABLE I

DEPOLARIZATION OF THE DESHEATHED FROG TIBIALIS NERVE BUNDLE

Depolarization by a Ringer's solution containing 20 mM K in the presence of lipid-soluble quaternary ammonium ions. Noracetylcholine 12 (Nach 12); pyridinium dodeciodide (PDI); pyridine-2-aldoxime dodeciodide (PAD). The effect on the depolarization is expressed as per cent compared with the control (= 100%).

Compound	*Concn. (M)*	*After 5 min exposure*	*On return to Ringer's solution*	
			5 min	*10 min*
Nach 12	10^{-4}	84%	85%	89%
PDI	10^{-4}	86%	86%	86%
PAD	10^{-5}	82%	63%	63%

Effect on permeability to Na

The change of permeability to Na, effected by lipid-soluble quaternary ammonium ions, was tested with concentrations of noracetylcholine 12 which produce a pronounced depolarizing effect in normal Ringer's solution. The concentrations are higher than those used in the experiments with K. When Na was replaced by choline chloride the depolarization was reduced by 30 to 50%. On again replacing choline chloride by Na the original degree of depolarization was restored (Fig. 6).

Dimethylaminoethyl acetate

Another way to mimic the action of acetylcholine and thus to test its functional importance on the Ranvier node is the application of its tertiary analog, dimethylaminoethyl acetate (DMAEA). It is known that this compound produces, at the myoneural junction, effects similar to those of acetylcholine although the concentrations required are about 100 times as high as with the quaternary compound. The tertiary analogue is hydrolyzed by acetylcholinesterase, although at a lower rate than the quaternary. The uncharged molecule is lipid-soluble; its pK is 8.2. The effect of DMAEA was tested in the absence of eserine and then in its presence, in order to find out whether a decreased rate of hydrolysis of the ester would increase its effect or would permit the use of smaller concentrations.

As might have been expected, the concentration required for marked effects was relatively high, $5 \cdot 10^{-3}\,M$. At this concentration without eserine the amplitude of the action potential is significantly increased, the spike height is greater, the descending phase is prolonged and only on further exposure the amplitude decreases. The first alterations appear within seconds. Even after more than 10 min exposure conduction is not yet blocked. If the nerve fiber is exposed to $5 \cdot 10^{-6}\,M$ eserine for 20 min, addition of $5 \cdot 10^{-3}\,M$ DMAEA produces complete block within 40 sec. On return of the fiber to Ringer's solution conduction is restored within 10 min (Fig. 7).

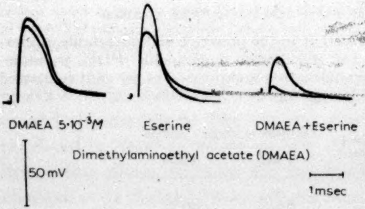

Fig. 7. Change in the mononodal action potential of a single nerve fibre preparation of the frog sciatic nerve (*Rana pipiens*) effected by dimethylaminoethyl acetate (DMAEA). 10 sec after the application of $5 \cdot 10^{-3}\,M$ DMAEA the spike is higher and the descending phase is prolonged. Eserine at a concentration of $5 \cdot 10^{-6}\,M$ has decreased the spike height after 25 min. Addition of DMAEA ($5 \cdot 10^{-3}\,M$) after exposure to eserine blocks conduction within 40 sec. When the fibre was returned to Ringer's solution the action potential had reappeared after 11 min although not yet to its full height. pH 7,7. Temperature, 23°.

DISCUSSION

If the action of acetylcholine on a receptor protein is responsible for the sudden change of Na conductance which takes place during activity, this process must be reversed within a fraction of a millisecond. The inactivation of acetylcholine is the function of acetylcholinesterase and the speed of this reaction and the enzyme concentration have been shown to satisfy the requirements. As a consequence of this view, conduction should become impossible if the enzyme activity is blocked. In spite of extensive investigations by NACHMANSOHN[1] and his colleagues which have shown that electrical activity is inseparable from the enzyme activity, DEL CASTILLO AND KATZ[3] objected to the evidence, because the concentration of eserine used in some preparations was several orders of magnitude higher than the one applied to junctions. This objection does not appear valid in view of the evidence that the minimum functional activity of the enzyme is independent of the outside concentration of the inhibitor, its structure and other factors; only the rate with which the minimum level required for inhibition is reached is affected, not the level itself. This excludes a non-specific effect implied in the criticism. The nature of the structural barriers surrounding the conducting membrane and the properties of the inhibitor will be one determining factor; in addition factors such as temperature, pH etc. must be of influence. The combination of all these factors makes it impossible to estimate the actual concentration of the inhibitor at the site of action. In previous reports by NACHMANSOHN and his colleagues 5 mg/ml of eserine were required to clock conduction in intact frog sciatic fibers in 30 min. However, on unmyelinated fibers, for example the squid giant axon, block may be achieved with 500 μg/ml, although in this case the period of time required is as long or even longer. (For a summary and evaluation of all these previous observations see NACHMANSOHN[2].) The data presented in this paper bring new evidence against the validity of the objections raised by DEL CASTILLO AND KATZ[3]. In frog sciatic nerve fat and connective tissue form a strong structural barrier. When a single nerve fiber is used where most of the barrier has been removed, block of conduction is obtained in less than 30 sec with 300 μg/ml of eserine, an increase in effectiveness of three orders of magnitude. The data provide an illustration for the correctness of the view that the outside concentration is irrelevant.

Another objection met by the observations reported is that raised by DAVSON[17] and also stressed by DEL CASTILLO AND KATZ[3] and other investigators. It was postulated that inhibition of the esterase should lead to an increase in spike amplitude and to a prolongation of the descending phase. If the action of acetylcholine continues because the speed of its removal is decreased, then obviously the increased Na conductance should not return within the usual time period to the resting level and an increased spike height and a prolongation of the descending phase should indeed be expected. This is exactly what has been observed on exposure of the Ranvier nodes to eserine in concentrations ranging from 1.5 to 15 μg/ml. In previous observations this particular point was not emphasized, since the aim of the earlier studies was the demonstration of the inseparability of enzyme activity and conduction; nevertheless, some of the records did show a definite prolongation of the descending phase[9]. However, in multi-fiber preparations such as the frog sciatic nerve containing many types of slow and fast conducting fibers, which may be affected at different time intervals and with different speeds, the response will be very complex. They are, therefore, not suitable for detecting very short-lasting and transitory effects as those described here with a single fiber preparation. Since cholinesterase is present approximately five times in excess of the minimum requirement, graded effects may be expected only in the neighborhood of the critical range of activity, *i.e.* 20 % of the initial. The zone of proper concentration may thus be very limited and the transition from an excess of enzyme to a level producing block may be very rapid and escape detection.

The remarkably strong potentiating effect of eserine on the action of DMAEA further supports the role of the acetylcholine system in electrical activity. Since eserine has such potent effects on the electrical activity of Ranvier nodes, eserine was added only at a very low concentration, 1.5 μg/ml. This is considerably lower than the conventional dose used at myoneural junctions. Nevertheless the action of the tertiary analogue of acetylcholine was potentiated by a factor of at least 15 fold or more.

The membrane potential in resting condition is, according to current concepts, essentially a result of the diffusion potential of K, Na and Cl ions. It may be formulated as follows (GOLDMAN[18]):

$$E = \frac{RT}{F} \ln \frac{P_K(K)_i + P_{Na}(Na)_i + P_{Cl}(Cl)_o}{P_K(K)_o + P_{Na}(Na)_o + P_{Cl}(Cl)_i}$$

where P_K, P_{Na}, P_{Cl} are permeability constants, $(K)_i$, $(Na)_i$, $(Cl)_i$ the concentration in the interior of the axon and $(K)_o$, $(Na)_o$, $(Cl)_o$ the concentration on the outside. The relative magnitude of the change in resting potential with a change in ion concentration provides a measure of the permeability to the ion. Decrease of cation effects indicates reduced permeability, whereas the contrary indicates increased permeability. A decrease in P_K alone will cause depolarization, an increase, hyperpolarization. The effect of an increment in K_o will be reduced or increased depending on whether P_K becomes greater or smaller. A change in the same direction in Na concentration will have the opposite effect on the resting potential from that of P_K because of the opposite direction of the Na gradient; hence an increase in P_{Na} depolarizes; a decrease, hyperpolarizes. Moreover it is apparent that as the Na concentration in the medium becomes lower depolarization due to an increase in P_{Na} may be reduced. Clearly, the sensitivity of the resting potential to changes in Na concentration of the milieu is directly related to the Na permeability. If this effect is increased or reduced by the action of a chemical compound, as in the present case by the lipid-soluble quaternary ammonium ions, the conclusion which follows is that these compounds produce a change in permeability for this ion relative to the other.

A solution containing 20 m*M* of KCl depolarizes to a lesser extent in the presence of the compound than in its absence. In view of the considerations outlined above, the effect indicates a decreased permeability to K, under the conditions of the test, since the depolarization caused by 20 m*M* KCl was smaller. If depolarization effected by the quaternary ions is caused by an increase of Na permeability, it should be absent or at least reduced in the absence of Na in the outer fluid. This is the case, as shown in Fig. 4.

The permeability of K, in the normal resting condition, is greater than that to Na and Cl and the membrane potential depends, therefore, essentially on the outside concentration of K. On exposure to the lipid-soluble quaternary ammonium ions the resting potential seems to be dependent on the external Na concentration; this is indicated by the degree and the rate of depolarization which occurs when the Na concentration is changed.

The selective change in permeability, *i.e.* a small but significant decrease to K together with a larger increase to Na, may change with the concentration of the compounds. By using higher concentrations of PAD the permeability for K was also increased. Measurements of the membrane resistance indicate that small concentrations of PAD, which cause only small depolarization, result in an increase in membrane resistance, possibly due to a decrease of permeability to K. With higher PAD concentrations which cause larger depolarization, the membrane resistance declines.

Further investigations are necessary to elucidate the changes of permeability to different ions effected by these quaternary ammonium ions as a function of their concentration and other factors of outer environment.

The effects of PAD further support the assumption that this quaternary ammonium ion acts upon the acetylcholine system. The theory that the action of acetylcholine on the receptor protein* is responsible for the sudden rise of Na conductance, assumes that the three proteins of the acetylcholine system directly associated with the elementary process — the storage protein, the receptor and the esterase — are located in very close vicinity within the conducting membrane. A quaternary ammonium ion with a dodecyl chain, such as PAD, could be expected to react with all three proteins due to a combination of Coulombic and the quite considerable Van der Waals' forces. The reported increase of permeability suggests an action on the receptor protein; a reaction with this protein *in vitro* has been observed by EHRENPREIS[20]. PAD is also a fairly strong acetylcholinesterase inhibitor in 10^{-6} M concentrations (GREENBERG[21]); moreover, the oxime is, in contrast to the physiological substrate, not hydrolyzable. Finally, it appears possible and even probable that these compounds may react with the storage protein and thereby displace bound acetylcholine. The effect may, therefore, be expected to be complex; much will depend on the relative affinities of PAD to these three proteins which at present are not known. Whereas the interpretation of the precise mode of action must remain open until more information is obtained, the data accumulated before[1,2] and reported here favor the conclusion that the effects of the quaternary ammonium ions must be attributed to an action upon the acetylcholine system. The evidence of this specific action is supported (a) by the demonstration of a competitive action with eserine, which is a potent inhibitor of cholinesterase as well as a receptor inhibitor[16]; (b) by the selective permeability change which is difficult to reconcile with an unspecific action producing a "hole" for ions[22,23], and (c) by the contraction of muscles obtained in the presence of high concentrations of curare (HINTERBUCHNER AND WILSON[24,25]), indicating that the compounds produce the biological effect attributed to the action of acetylcholine. The presence of an acetylcholine system and the analogy of structure between the quaternary ammonium ions and acetylcholine make the interpretation proposed a likely one.

ACKNOWLEDGEMENTS

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* EHRENPREIS[19] has recently isolated a protein from electric tissue of electric eel which exhibits receptor-like properties in that it reacts with a variety of neurotropic agents including acetylcholine, curare, eserine and PAD.

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AGGREGATION OF MYOSIN A

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SUMMARY

Myosin A molecules aggregate and form polymers of about 1 μ in length, when the ionic strength or pH of their solution is lowered. In 0.2 M KCl, myosin A is fully dissociated at pH 7.3 and higher, but fully polymerized at pH 6.5 and lower.

When myosin A solution is in an intermediate state of aggregation, addition of ATP causes dissociation.

The birefringence of myosin A solution corrected to full orientation and unit concentration is $1.2 \cdot 10^{-2}$.

INTRODUCTION

Myosin A, or simply myosin, which is one of the representative component proteins of muscle, dissolves to give a clear solution in a medium of aqueous KCl approx. 0.5 M. In the course of determining the molecular weight of myosin A in a high ionic strength medium of this type, various measured values indicated that myosin A had a tendency

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Abbreviations used: ATP, adenosinetriphosphate; χ, extinction angle; G, gradient of flow, Δn, birefringence; f, orientation factor; c, concentration of solute; θ, rotary diffusion constant.

to aggregate[1-9]. The painstaking work of JOLY *et al.*[10] covered the pH range 6 to 8, in 0.5 M KCl and showed the presence of dimers and trimers of myosin A. When the pH or ionic strength of the solution is lowered, the myosin A tends to aggregate. SPICER *et al.*[11] studying the aggregation of myosin A, discovered many interesting facts, and suggested the importance of the role of myosin A aggregation in the mechanism of muscle contraction. MORALES *et al.*[12] assumed the presence of a polymer of myosin A in a solution of myosin B in 0.6 M KCl in their attempt to interpret the results of light scattering and sedimentation experiments.

The technique of flow birefringence measurement is very effective in the determination of molecular size, especially for determining the length of molecules of fibrous proteins. Thus a survey of the change of particle size in a myosin A solution over a range of pH, 5.5 to 7.5, and of ionic strength, 0.02 to 0.6 M, was made from the view-point of flow birefringence.

METHODS

Preparation of myosin A

Myosin A was prepared according to the usual method although with slight modifications. Minced skeletal muscle of a rabbit was extracted with Guba-Straub solution for 10 min. The extract was first diluted to 0.15 M with respect to KCl concentration, and the precipitate was removed by centrifugation. Then the solution was diluted to 0.05 M, and the precipitate was collected and dissolved in 0.6 M KCl. The same procedure of dilution, precipitation and dissolution was repeated once or twice more. The final precipitate was dissolved in 0.6 M KCl at a concentration of about 2 %. When this was to be stored for a period of one night or more, an equal volume of glycerin was added and the mixture was placed in the freezing compartment of a refrigerator. During the preparation time and before measurement the temperature of the solution was kept low in an ice-water bath.

The concentration of the stock solution was determined by the micro-Kjeldahl method. The amount of birefringence of the solution in 0.2 M KCl at pH 6 and at a flow gradient of 1000 sec^{-1} was also used for the estimation of the concentration.

Flow birefringence measurement

The apparatus was an EDSALL type[13], manufactured by Rao Instrument Co. A black teflon cell was used, and the myosin A solution was in direct contact only with teflon and glass. During the measurement, the temperature of the solution was kept at 20 ± 1° by circulating water from a thermostat.

The stock solution of myosin A, kept at the temperature of ice, was diluted to give a solution of desired concentration, pH and ionic strength, immediately before the measurement. It was then immersed in a water bath of 20° for several minutes, degassed under vacuum, and placed in the flow birefringence apparatus.

pH measurement

The pH of the solution was adjusted by the addition of potassium phosphate buffer of suitable pH to a final concentration of 0.02 M. After each flow birefringence measurement, the pH of the solution was measured with a Beckman model G glass electrode pH meter. In most cases, the pH was also measured before and after the addition of ATP to the myosin A solution, and the ATP addition was found to cause a change of pH of no more than 0.02 units.

RESULTS

The results of the flow birefringence measurements on myosin A in 0.6 *M* KCl were as shown in Fig. 1. There was no tendency for the extinction angle (χ) to become low at lower gradients of flow *(G)*, and the angle clearly extrapolated to 45° at zero gradient as was expected by the theory. This result is slightly different from that reported previously[15] in which the angle of extinction was lower for lower values of *G*. The length of the myosin A molecule calculated from this result was 1700 Å, which agrees well with the results obtained by the light scattering method[4,8,9]. ATP had no effect on the flow birefringence properties of myosin A in 0.6 *M* KCl. The pH had no effect on the molecular size of myosin A at this high ionic strength, except at a very low pH of about 5.5 where isoelectric precipitation sets in.

With various ionic strengths of the solution, the values of the rotary diffusion constant of myosin A were as shown in Table I.

TABLE I

ROTARY DIFFUSION CONSTANT AND LENGTH OF MYOSIN A POLYMERS AT VARIOUS IONIC STRENGTHS

KCl concentration (M)	*Myosin A concentration (%)*	*pH*	*θ(sec^{-1})*				*Particle length (μ)**			
			G = 0	*10*	*100*	*1000*	*G = 0*	*10*	*100*	*1000*
0.6	0.13	7.4	3800	—	—	3600	1700 Å	—	—	1700 Å
0.3	0.15	5.9	—	2.5	13	37	—	1.9	1.1	0.8
0.2	0.006	6.2	11	12	18	22	1.2	1.1	1.0	0.9
0.1	0.02	6.2	2.4	3.0	6.0	11	2.0	1.8	1.4	1.2
0.02	0.02	7.4	10	11	19	47	1.2	1.2	1.0	0.7

* The length of the prolate ellipsoid was calculated from the formula of PERRIN[16], with a postulated axial ratio of 100[17].

In 0.3 *M* KCl myosin A was monomeric* at pH 6.4 or higher, and there was a tendency for end to end aggregation at lower pHs. However, the degree of polymerization was far lower than that obtained in 0.2 *M* KCl which is described in the following, and flow birefringence measurement was hardly possible at a concentration of 0.01 %.

In 0.2 *M* KCl, myosin A showed a quite different flow birefringence behavior. At pH 6.2, a typical relationship of the extinction angle and the birefringence (Δn) with the flow gradient is shown in Figs. 2 and 3. The extinction angle was independent of the concentration of myosin A over the range 0.002 to 0.01 %. In a solution of higher concentration, χ was slightly less in the lower *G* range indicating the presence of intermolecular interaction. The rotary diffusion constant (θ) calculated from the initial slope of the χ-G curve was 10 sec^{-1}. The molecular aggregates of myosin A in 0.2 M KCl at pH 6.2 were about 1 μ in length. The χ and Δn stayed at the same value when the pH was raised to 6.5.

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* Since the rotary diffusion constant is not sensitive to change of molecular width, the word "monomeric" used here only denotes that the length of the molecule or particle is that of a single molecule. A possible lateral association[8,9] cannot be observed by the flow birefringence technique.

At pH 6.9 the extinction angle was quite different to that at a lower pH. The results for pH 6.9 are shown in Fig. 4. The χ increased when G increased from 500 to 2000 sec^{-1}. The χ was dependent on concentration at 0.05 % which was the lowest possible concentration at which flow birefringence measurements could be carried out due to the low Δn at this pH. The most remarkable fact of all was that the χ and Δn were clearly affected by the addition of ATP. The χ increased while Δn was reduced to about half its value. At a pH of 7.3 in 0.2 M KCl, the flow birefringence behavior of myosin A was just the same as that in 0.6 M KCl.

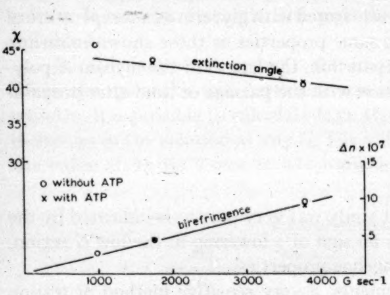

Fig. 1. Extinction angle and birefringence of a myosin A solution of 0.13 % in 0.6 M KCl at pH 7.4.

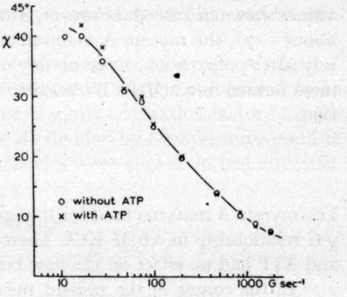

Fig. 2. Extinction angle of a myosin A solution of 0.006 % in 0.2 M KCl at pH 6.2.

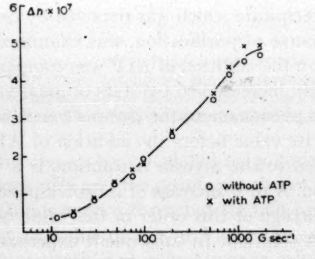

Fig. 3. Birefringence of a myosin A solution of 0.006 % in 0.2 M KCl at pH 6.2.

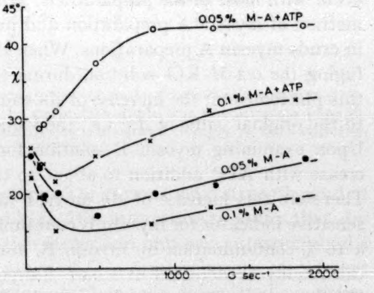

Fig. 4. Extinction angle of myosin A solutions in 0.2 M KCl at pH 6.9.

ATP addition had no effect on the flow birefringence properties of the myosin A solution in 0.2 M KCl, except at pH 6.9 where myosin A was in an intermediate state between full polymerization and full dissociation. At a pH of about 6, the Δn of myosin A solution sometimes increased upon addition of ATP, but this was found to be caused by contamination by myosin B. This matter will be discussed in more detail later.

In 0.1 M KCl, myosin A was no longer monomeric even at pH 7.3, and at pH 6.9 it was almost completely polymerized. In 0.02 M KCl, myosin A was polymerized even at pH 7.3, and in the lower pH region, coagulation and precipitation took place when the solution was kept in a field with a velocity gradient and flow birefringence measurement was impossible.

When myosin A stock solution was stored at 0° over night without glycerol, the size of the polymerized particles in 0.2 M KCl at pH 6.2 was slightly longer than the values shown in Table I. However, when it was stored with glycerin at a temperature of about —7°, the myosin A maintained the same properties as those shown immediately after preparation. Even on day of preparation, the length of the myosin A polymers formed in 0.2 M KCl tended to increase with the passage of time after preparation.

DISCUSSION

The myosin A material used for the present study was very pure as is indicated by the χ-G relationship in 0.6 M KCl. There was no sign of χ lowering in the low G region, and ATP had no effect on the flow birefringence properties.

In the course of the present measurements, a very sensitive method of testing the purity of myosin A was found. In a few preparations of myosin A, a rise of Δn was caused by the addition of ATP to myosin A in 0.2 M KCl at pH 6.2, but this did not occur with most of the preparations. When this phenomenon was traced back to the method of myosin A preparation and purification, it was found to be more marked in crude myosin A preparations. When the precipitate which was removed by centrifuging the 0.2 M KCl solution during the course of purification, was examined for this phenomenon, the increase of Δn caused by the addition of ATP was comparable to the original value of Δn, *i.e.*, the value of Δn increased to twice its original value. Upon examining myosin B solution for this phenomenon, the Δn was found to increase with ATP addition to about 10 times its value before the addition of ATP[14]. Therefore this increase of Δn on ATP addition to the myosin A solution, is a very sensitive indicator for myosin B contamination. A 10 % increase of Δn corresponds to a 10 % contamination by myosin B, and a change of this order in the value of Δn can readily be detected in a 0.01 % myosin A solution. In subsequent experiments, all the preparations used were examined for myosin B contamination by use of this method.

In 0.2 M KCl at pH 6.2, myosin A was in a polymerized condition, and the flow birefringence properties were as shown in Figs. 2 and 3. When θ and the molecular length were calculated for various values of G, the results were as shown in Table I. The values in Table I were calculated from χ for myosin A solutions with such concentrations that χ was independent of concentration, and therefore they may be taken to be the values for free particles in an infinitely dilute solution. When the concentration was higher, χ was lower, and the apparent θ calculated was smaller. However, the difference was not very significant; at the highest possible concentration which allowed flow birefringence measurement, 0.04 %, the apparent θ values were only about half the corresponding values for a dilute myosin A solution. This means that the degree of concentration dependence of the χ was small, and thus indicates that the intermolecular interaction between polymerized myosin A particles was small.

The most remarkable aspect for the myosin A polymerization is the small range of variation in the calculated particle length for different values of G. This is in contrast to the results obtained for myosin B in 0.6 M KCl (see ref. 15) in which θ at $G = 1000 \text{ sec}^{-1}$ was more than 10 times that calculated for $G = 0$. The small variation in the calculated θ for a myosin A polymer over a wide range of G values indicates that the size of the polymerized particles of myosin A is relatively uniform. Within the range of the present study, the size of polymerized myosin A was longer for lower pHs and for lower ionic strengths although the difference was very small as can be seen in Table I.

Another fact worth noting is the amount of birefringence of myosin A solution. When the molecular and particular size in the myosin A solution is assumed to be uniform, it is possible to calculate from the value of χ, the orientation factor f for the molecules in the solution at any G. The value of Δn divided by f would correspond to the value of Δn for a case in which all the molecules were aligned in one direction.

TABLE II

BIREFRINGENCE OF MYOSIN A

KCl concentration (M)	*Myosin A concentration* (%)	*pH*	$(\Delta n/f.c)^* \times 10^2$	
			—ATP	*+ATP*
0.6	0.13	7.4	1.3	1.4
0.2	0.006	6.2	1.2	1.2
0.2	0.024	6.2	1.2	1.3
0.2	0.05	6.5	1.0	1.1
0.2	0.1	7.3**	0.8	1.5
0.1	0.02	6.2	1.4	1.1
0.02	0.02	7.4	1.2	1.0

* The amount of birefringence corrected to full orientation of molecules and unit concentration.
** With these conditions, the measurement of the extinction angle was not accurate enough. The orientation factor is small and changes very sensitively with small changes in the extinction angle when the extinction angle is close to 45°.

Such values were calculated for myosin A solutions of various ionic strengths and at various pHs and the results are shown in Table II. It is interesting to notice that $\Delta n/f.c.$ (birefringence corrected for unit orientation factor and unit concentration) had a constant value of $1.2 \cdot 10^{-2}$ over the entire range of G and for solutions of different ionic strength if myosin A was in a fully polymerized or fully dissociated condition. The fact that the value of $\Delta n/f.c.$ was almost constant indicates that the first assumption of uniform particle size was not far from the truth. This supports the conclusion regarding the uniform size of myosin A polymers deduced from the χ–G relationship. On the other hand, when myosin A was in a transient condition, such as in 0.2 M KCl at pH 6.9, the value of $\Delta n/f.c.$ was lower than $1.2 \cdot 10^{-2}$, and changed with variations in G. In such an intermediate condition, the χ–G relationship showed a shape typical of a mixture* (Fig. 4). The χ stayed constant over the middle G range, and then increased when G was raised to a high value of 1000 sec^{-1} or higher. The low value of $\Delta n/f.c.$ for the myosin A solution in an intermediate state of polymerization indicates that

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* *Note added in the proof*: When a rough calculation was performed by the formula of SADRON[18] while assuming that there were only particles of two sizes with θ of 20 and 3800 sec^{-1}, the 0.1 % myosin A solution of Fig. 4 contained 4 % of larger particles and the 0.05 % solution contained only 0.2 %, both in the absence of ATP.

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the particle length spectrum for such a myosin A solution is very broad and possibly two peaks are at the fully polymerized and fully dissociated positions.

In 0.2 M KCl at pH 6.9, addition of ATP had a remarkable effect on the flow birefringence of myosin A solution. The rise of χ and the decrease of Δn clearly indicated that the number of large particles decreased and the number of small ones increased, *i.e.*, ATP caused the dissociation of the myosin A polymer. However, since the transition from the fully polymerized state to the fully dissociated state took place when there was a small change in pH, even of less than one unit, it was suspected that the real cause for the dissociation was the change of pH caused by the addition of ATP. The pH of the solution was measured carefully with a glass electrode pH meter, and it was found that the addition of ATP caused a change of pH of less than 0.02 units. Therefore it was verified that the dissociation of myosin A polymer was really caused by the addition of ATP.

This phenomenon of dissociation of the myosin A polymer has, perhaps, something in common with the assumption made by MORALES *et al.*[19] that the 5-h myosin B contained a considerable fraction of myosin A polymer which had dissociated under the influence of ATP. However, it should also be noted that the dissociation of the myosin A polymer by ATP takes place only under very restricted conditions, and that the flow birefringence properties of a mixture of myosin A and myosin B are the additive sum of those of each component.

As was pointed out by SPICER[11], the change in the state of myosin A caused by pH, ionic strength and ATP addition in a solution of roughly physiological condition, should have a close connection with the mechanism of muscular contraction. The fact that a small change in pH of less than one unit causes the full dissociation of polymerized myosin A, and the fact that ATP causes dissociation of the myosin A polymer when it is at a pH and ionic strength of partial dissociation, are very interesting. Clarification of the mechanism of polymerization and dissociation of myosin A molecules would be useful for the elucidation of the role of myosin A and myosin B in the contraction of muscle.

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EFFECT ON MYOSIN B OF KCl CONCENTRATION AND pH

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SUMMARY

Flow birefringence measurements were performed on solutions of myosin B from rabbit skeletal muscle, over a KCl concentration range 0.1 to 0.6 *M* and a pH range 6 to 8.

When the concentration of KCl was higher than about 0.3 *M*, the flow birefringence properties of myosin B solution both with and without ATP was roughly the same as those in 0.6 *M*.

At lower KCl concentrations, the myosin B solution became turbid, and showed weak birefringence. Upon addition of ATP to such a solution, a clear phase formed and the solution became strongly birefringent. The molecular size derived from the extinction angle under such conditions was about the same as that in 0.6 *M* KCl with ATP.

The amount of birefringence in the clear phase was dependent on the KCl concentration and pH. The concentration of KCl at which birefringence was maximum decreased as the pH of the solution was raised. At pH 7, birefringence was maximum at 0.15 *M* KCl, which is roughly the physiological condition.

The elucidation of the molecular condition of myosin B in the clear phase may furnish important evidence for determining the mechanism of super-precipitation.

INTRODUCTION

Myosin B is generally accepted as playing the principal role in the contraction of muscle[1]. In this respect, the properties of a solution of myosin B, especially its interaction with ATP*, have been studied by numerous workers using all sorts of methods. The technique of flow birefringence has been applied to the study of the interaction between myosin B and ATP, and some experimental facts which are of some use for the solution of the problem have been reported previously[2].

Almost all the measurements hitherto performed on myosin B have been in a medium of relatively high ionic strength, *i.e.* around 0.6 *M* KCl. However, it is known that the effect of ATP on myosin B is different, when the ionic strength of the solution is changed. When the concentration of KCl is high, about 1 *M*, the properties of myosin B in this solution are similar to those in 0.6 M KCl in the presence of ATP, and thus ATP has no effect on myosin B in a solution of such high salt concentration[2]. When the ionic strength of the solution is low, myosin B tends to form a flocculent suspension and super-precipitation is caused by the addition of ATP.

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* The following abbreviations are used. ATP, adenosinetriphosphate; θ, rotary diffusion constant; χ, extinction angle; Δn, birefringence; G, velocity gradient of flow; PP, pyrophosphate; f, orientation factor; c, weight concentration, g/ml.

Since examination of the effect of ionic strength on the molecular shape and size of myosin B seemed to be of value for the elucidation of the interaction between myosin B and ATP, flow birefringence measurements were attempted over a wide range of KCl concentration.

When the concentration of KCl was 0.3 M or higher, myosin B dissolved to give a clear solution, and the flow birefringence properties were roughly the same as in 0.6 M KCl. The effect of ATP in such a solution was, in brief, the reduction of the birefringence. When the ionic strength was lowered, the solution of myosin B became turbid and often there was visible flocculation, although even if such a solution was left to stand for several hours no significant precipitation could be observed. When ATP was added to such a solution, super-precipitation took place. If Mg^{++} was also present, the solution became clear on ATP addition[3], and remained clear until super-precipitation set in. In this clear phase, the solution showed remarkable streaming birefringence.

It was found that in the clear phase the rotary diffusion constant (θ) of the myosin B molecule was about the same as in 0.6 M KCl in the presence of ATP, and thus the molecular shape and size of myosin B and its interaction with ATP in 0.6 M KCl is directly connected with the interaction of myosin B with ATP under physiological conditions.

EXPERIMENTAL

Myosin B preparation

Rabbit skeletal muscle was minced, and quickly homogenized for 10 sec in a Waring blendor with 3 volumes of Weber-Edsall solution. The suspension was placed for 24 h in a refrigerator, then squeezed with the hands after the addition of a half volume of 0.6 M KCl. The KCl concentration of the extract was reduced to 0.2 M by dilution with water, the precipitate was collected by centrifugation, and the sediment was dissolved in 0.6 M KCl. The fibrous muscle debris and other insoluble matter was removed by centrifugation at a KCl concentration of 0.4 M. The dilution and precipitation in 0.2 M KCl and dissolution in 0.4 M KCl were repeated 3 times, and then the final solution was clarified by centrifugation for one hour at 12,000 × g.

Flow birefringence measurements

The apparatus and the procedure for measurements have been described elsewhere[2]. The temperature was kept at 20° during the measurement. The pH of the solution was adjusted to the desired value by the addition of phosphate buffer solution to give a final phosphate concentration of 0.02 M. The crystalline ATP obtained from Sigma was dissolved and neutralized just prior to the measurements.

RESULTS

Effect of pH and the KCl concentration on the flow birefringence properties of myosin B

Myosin B dissolved and gave a clear solution when the concentration of KCl was higher than about 0.3 M. When the KCl concentration was reduced below a certain limit, which depended on the pH of the medium, the solution became turbid, and the measurement of the birefringence, especially of the extinction angle (χ), became difficult. Even so, the amount of birefringence (Δn) could be determined with fairly good accuracy, and the value was about half of that obtained in 0.6 *M* KCl. In the range of KCl concentration below this limit, the clear phase occurred, *i.e.* Δn increased upon addition of ATP, and the value was very dependent on the KCl concentration.

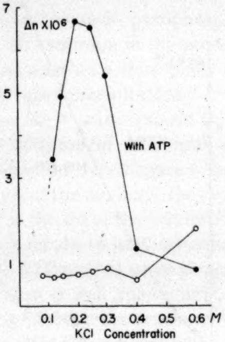

Fig. 1.

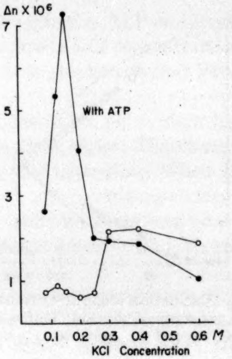

Fig. 2.

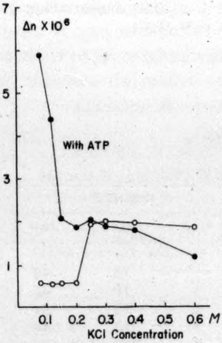

Fig. 3.

Fig. 1. Flow birefringence at $G = 1970$ sec^{-1} for a solution of myosin B of 0.1 %, at pH 6.1 and with 1.5 m*M* $MgCl_2$.

Fig. 2. Flow birefringence at $G = 1970$ sec^{-1} for a solution of myosin B of 0.1 %, at pH 7.1 and with 1.5 m*M* $MgCl_2$.

Fig. 3. Flow birefringence at $G = 1970$ sec^{-1} for a solution of myosin B of 0.1 %, at pH 7.6 and with 1.5 m*M* $MgCl_2$.

At pH 7 in the clear phase, Δn reached a sharp maximum value at around 0.15 *M* KCl, which is roughly the physiological condition. The concentration of KCl giving this maximum value of Δn, increased when the pH of the medium was reduced. Results of such measurements at pH 6.1, 7.1 and 7.6 are shown in Figs. 1, 2 and 3 respectively.

We used 1.5 m*M* of Mg^{++} for the purpose of extending[4] the period of the clear phase, and it was about 0.5 h to 1 h before the super-precipitation set in. Typical super-precipitation took place after the birefringence measurement, when the solution was left to stand in a test tube at the room temperature.

χ and Δn were measured very accurately in the clear phase. A typical result for the relation of χ and Δn to the velocity gradient of flow (G) is shown in Fig. 4. The rotary diffusion constant calculated from the measured values of χ at different KCl concentrations and pH 7 are shown in Table I. Almost the same results were obtained with other pH values. As is seen in the table, θ for a myosin B molecule in the presence of ATP remains almost independent of the KCl concentration.

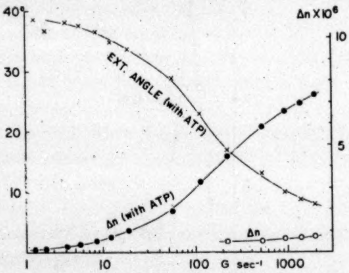

Fig. 4. Extinction angle and birefringence of flow for a solution of myosin B of 0.1 %, in 0.15 M KCl at pH 7.1, and with 1.5 mM $MgCl_2$.

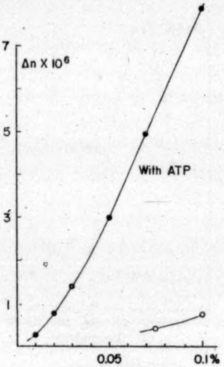

Fig. 5. Dependence of flow birefringence at $G = 1970\ sec^{-1}$ on the concentration of myosin B in 0.15 M KCl at pH 7.1 with 1.5 mM $MgCl_2$.

TABLE I

ROTARY DIFFUSION CONSTANT OF A MYOSIN B MOLECULE

pH = 7.1 and 1.5 mM $MgCl_2$.

KCl concentration	*Rotary diffusion constant (sec⁻¹)*					
	Without ATP			*With ATP*		
	G = 10	*100*	*1000*	*G = 10*	*100*	*1000*
0.12	—	—	—	—	15	24
0.15	—	—	—	5	16	22
0.20	—	—	—	4	13	34
0.25	—	—	—	3	12	42
0.3	2	5	19	3	14	43
0.4	2	5	20	3	12	45
0.5	2	6	18	3	17	59
0.6	2	5	15	4	13	51

Effect of protein concentration

In the clear phase at pH 7.1 and 0.15 M KCl, Δn was measured over a range of protein concentrations, and it was found that Δn was roughly proportional to the protein concentration as can be seen in Fig. 5. The deviation from linearity of the curve at very low protein concentrations of less than 0.01 % seems to indicate some kind of dissociation[5] which occurs on high dilution.

Effect of ATP

In the range of KCl concentration over which the clear phase occurred, an ATP concentration of 0.5 m*M* was sufficient to cause the clear phase to occur and for Δn to increase. When the concentration of ATP was raised, there was no further change in Δn, but the length of the period of the clear phase was elongated[4].

Effect of other reagents

As is well known[2], pyrophosphate (PP) has the same effect as ATP on myosin B in 0.6 *M* KCl solution in the presence of Mg^{++}, but at a lower KCl concentration PP did not cause either a clear phase or an increase in Δn. It is also known that PP does not cause super-precipitation.

When Mg^{++} was replaced by Ca^{++}, super-precipitation took place immediately after the addition of ATP with a scarcely observable clear phase. Therefore, any measurement of birefringence during the clear phase was impossible. When Mg^{++} was left out of the medium, the clear phase was very short, and no striking change was observed in the Δn of the solution before and after ATP addition. There was, however, a tendency for Δn to have a minimum at about a KCl concentration of 0.2 *M*, and at this point ATP caused some increase in Δn.

Salyrgan, a well known inhibitor for myosin B adenosinetriphosphatase, had an interesting effect on the action of ATP. For myosin B in 0.6 *M* KCl, salyrgan enhanced the effect of ATP, regardless of the time of addition before or after ATP. In the lower KCl concentration range, however, salyrgan completely inhibited the action of ATP causing the clear phase and super-precipitation, if salyrgan was added before ATP. When salyrgan was added after the addition of ATP, Δn decreased, but only to about a third of its original value which is still a few times greater than the value for myosin B before the addition of ATP (*cf.* Table II).

TABLE II

EFFECT OF SALYRGAN ON THE MYOSIN B–ATP SYSTEM

Myosin B concentration, 0.1 %; temperature, 20°; $G = 1970\ sec^{-1}$.

Additives (Added in the order designated by +)	*Flow birefringence results*			
	0.6 M KCl, pH 7.1		*0.2 M KCl, pH 6.1*	
	χ	Δn ($\times 10^7$)	χ	Δn ($\times 10^7$)
Myosin B	8.7°	18.4	—	6.3
Myosin B + ATP	13.3	10.0	9.3°	74.8
Myosin B + ATP + salyrgan	16.1	6.3	—	24.9
Myosin B + salyrgan	17.0	6.5	—	7.7
Myosin B + salyrgan + ATP	21.0	4.6	—	6.6

DISCUSSION

When the results of the measurements over the range of KCl concentration and pH studied in the present experiment were carefully considered as a whole, the following two points drew attention. First, the rotary diffusion constant of a myosin B molecule or its aggregate and the change of θ with the flow gradient remained constant regardless of the concentration of KCl both in the presence and absence of ATP (*cf.* Table I).

This constancy indicates that the distribution of the length of the molecules of myosin B which contribute to the flow birefringence of the solution was not affected by the change of pH or of KCl concentration.

The second point was that the amount of birefringence occurring for a given protein concentration was a function of pH and KCl concentration. In addition, ATP had a profound effect: when myosin B was in a clear solution the amount of birefringence decreased, and when it was in a turbid suspension the amount of birefringence increased. The former effect has been well known since the work of NEEDHAM *et al.*[6], and was studied in detail in our previous work[2]. The latter effect of ATP in increasing the birefringence of myosin B at lower KCl concentrations, apparently is closely related to the effect of ATP in causing the clear phase which precedes super-precipitation.

As seen from the first point mentioned above and as described in Table I, the size distribution for the myosin B molecules and its aggregates in the clear phase was about the same as in 0.6 M KCl solution in the presence of ATP. There is, however, a slight difference between, for example, 0.15 and 0.6 M. θ calculated at $G = 1000\ \text{sec}^{-1}$ decreases from about 50 to 20 sec^{-1} as the concentration of KCl changes from 0.6 to 0.15 M. This is perhaps because myosin A exists to a considerable extent in the solution of myosin B with ATP, and because myosin A is in the highly dispersed condition of $\theta = 3800\ \text{sec}^{-1}$ in 0.6 M KCl (see ref. 2), and exists as end to end polymers of θ of about 10 sec^{-1} in 0.15 M KCl (see ref. 7). The greater value of Δn at lower KCl concentration than 0.6 M both in the presence of ATP may also be explained by the formation by myosin A of aggregates of a size suitable for contribution to the birefringence of the solution.

In contrast to the independence of θ for the myosin B molecule of the change of KCl concentration and pH, θ varied considerably for different G values. This is perhaps mainly due to the wide range of distribution of the molecular length. In 0.6 M KCl, it was verified that myosin A of $\theta = 3800\ \text{sec}^{-1}$ formed under the influence of ATP from myosin B of θ of about 1 sec^{-1} (see ref. 2, 8). Therefore, the large θ value of myosin B in the presence of ATP at $G = 1000\ \text{sec}^{-1}$ is partly due to the presence of myosin A. In 0.15 M KCl at pH 7, myosin A is expected to form aggregates, and θ would be about 20 sec^{-1} for $G = 1000\ \text{sec}^{-1}$ (see ref. 7), and thus the presence of myosin A should not have much effect on the value of θ of myosin B in 0.15 M KCl at $G = 1000\ \text{sec}^{-1}$. Thus the variation of θ for different G in solutions of lower KCl concentration should be due to the size distribution of myosin B. However, since θ varies proportionally to the third power of the molecular length as is shown in Table III,

TABLE III

ROTARY DIFFUSION CONSTANT AND MOLECULAR LENGTH

θ	$2a$*
3	1.8 μ
5	1.5 μ
10	1.2 μ
30	0.9 μ
50	0.7 μ

* The length was calculated[9] by assuming the molecules to be rigid ellipsoids with an axial ratio of 100 (see ref. 10). The deviation of the axial ratio by a factor of 10 would change the length by less than 20%.

the range of distribution is not as wide as it seems. From the values in Table I, it is assumed that the longest of the molecular aggregates of myosin B is about three times as long as the shortest member.

The values of χ and Δn in the clear phase indicate that myosin B is solubilized and dispersed by the action of ATP. It is interesting to compare this with the state of aggregation of myosin A at such low KCl concentration[7]. This kind of molecular dispersion of myosin B seems to be a prerequisite for the occurrence of super-precipitation. SPICER[3] first noticed that the clear phase was an essential precursor of super-precipitation, but he was of the opinion that ATP did not cause the dissociation of synthetic actomyosin in the clear phase[4]. As regards the molecular mechanism of super-precipitation, very little is known so far, but as was suggested by SPICER[3], some kind of intermolecular bridge formation seems to play an important role. Myosin B does not dissolve in 0.15 *M* KCl solution, but forms large aggregates which scarcely contribute to the birefringence of the solution. ATP causes such aggregates to disintegrate into smaller particles which show birefringence. Such a condition of molecular aggregation and dispersion may be necessary for bringing myosin B into a rearranged state of super-precipitation. Recently LEVY AND KOSHLAND[11] have presented evidence for a phosphorylated intermediate from actomyosin in the clear phase preceding the superprecipitation. There is a possibility that this intermediate corresponds to the dispersed molecules observed by flow birefringence.

It is of special interest here to consider the absolute amount of birefringence occurring in muscle structural proteins. In conditions in which Δn of myosin B has a maximum value, *i.e.* about 0.05 *M* KCl at pH 7.6, 0.15 *M* KCl at pH 7.1, and 0.2 *M* KCl at pH 6.1 in the presence of ATP, the amount of Δn calculated for full orientation of the molecules and for unit concentration (g/ml), $\Delta n/f.c$, was always around $1.2 \cdot 10^{-2}$. When such values were calculated for other kinds of muscle structural proteins such as actin, myosin and tropomyosin from different animals, an almost constant value of $1 \cdot 10^{-2}$ was obtained as is shown in Table IV. This fact is of much importance, since the same value was observed for the actomyosin thread by WEBER[12].

TABLE IV

FLOW BIREFRINGENCE OF MUSCLE PROTEIN

Protein	*Animal*	$\Delta n/f.c$ ($\times 10^2$)
Myosin B in 0.6 *M* KCl	Rabbit	0.3
	Honeybee	0.6
	Crayfish	0.1
	Earthworm	0.2
	Clam	0.1
Myosin B + ATP in 0.15 *M* KCl	Rabbit	1.2
	Honeybee	1.4
Myosin A in 0.6 *M* KCl	Rabbit	1.2
	Honeybee	1.0
	Crayfish	1.0
	Earthworm	0.6
F-actin in 0.1 *M* KCl	Rabbit	~ 1.0
	Honeybee	0.5
Tropomyosin in water at pH 6.4	Rabbit	1.0
	Crayfish	1.0

It should, however, be noted that $\Delta n/f.c$ for myosin B in 0.6 *M* KCl was definitely smaller than the value above, and decreased further upon addition of ATP. The co-existence of myosin A with myosin B as a small contamination in most myosin B preparations and as the product formed by the influence of ATP, seemed to be the main cause of this low value of $\Delta n/f.c$. As is clear from the theory of SADRON[13], if $\Delta n/f.c$ is calculated by assuming a mixture to be a simple substance, a low value is to be expected. Upon performing an actual calculation, this effect was found to be not sufficient to give a full explanation. There is a possibility that the optical factor of the myosin B molecule in 0.6 *M* KCl is different from that in lower KCl concentrations.

Another interesting fact found in the course of the present study was that around the KCl concentration of 0.3 *M*, where the flow birefringence properties of myosin B are intermediate between those for 0.6 and 0.15 *M*, the relation of χ to G for myosin B from rabbit was very similar to that for myosin B from honeybee[14] in 0.6 *M* KCl. At such a point, the addition of ATP caused Δn to decrease in the low G range, but in the higher G range Δn did not decrease appreciably and sometimes even increased upon addition of ATP. It has been difficult to explain such behavior of the myosin B from honeybee on the basis of the molecular mechanism assumed for the interaction of rabbit myosin B with ATP in 0.6 *M* KCl, and therefore the insect myosin B was supposed to be unusual in this respect. However, since the same phenomenon was observed for rabbit myosin B, and since it is now known that such a phenomenon is intermediate between the dissociation in the high salt concentration range and the clear phase phenomenon in the low salt concentration range, there is no longer any need to regard honeybee myosin B as unusual.

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A MODEL FOR THE MYOSIN MOLECULE

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SUMMARY

The molecular weight of myosin has been determined in 0.5 M KCl and 5 M guanidine·HCl.

1. The molecular weight, using the Archibald approach to equilibrium method, is strongly dependent on protein concentration in both solvents giving infinite dilution values of $6.19 \cdot 10^5$ in 0.5 M KCl and $2.06 \cdot 10^5$ in 5 M guanidine·HCl.

2. Sedimentation, diffusion and viscosity studies on myosin in 5 M guanidine·HCl provide estimates of the molecular weight in reasonable agreement with the Archibald studies.

3. Preferential binding of guanidine·HCl by myosin has been determined and shown to be of the order of 5 % or less.

4. Utilizing these molecular weight determinations and available X-ray diffraction, light scattering and optical rotatory data, a model for the myosin molecule is proposed, based on three equal weight polypeptide chains in the form of a three-stranded α rope.

INTRODUCTION

It has been evident for several years from the work of WEBER AND STÖVER[1], SZENT-GYÖRGYI[2], MOMMAERTS[3] and, particularly, from the work of TSAO[4] that the myosin molecule is dissociated into smaller units through the action of the hydrogen bonding agents, urea and guanidine·HCl. From his osmotic pressure measurements in urea solution, TSAO[4] concluded that the principal component in myosin preparations was a polypeptide chain with a molecular weight of 165,000.

It has also been proposed[5] that myosin is composed of subunits dissociated by a number of proteolytic enzymes. While it has been assumed in the past that these units enjoy a separate existence, it is now known[6] that their dissociation involves the rupture of a large number of peptide bonds. This detracts somewhat from the original proposal; nevertheless, this unique action of proteolytic enzymes and the properties of the fragments provide valuable keys to the structure of myosin.

The present report is the result of extensive studies on the behavior of myosin in concentrated solutions of guanidine hydrochloride utilizing the Archibald sedimentation equilibrium technique, supported by velocity sedimentation, diffusion and viscosity studies. It also includes the assessment of ancillary factors such as the binding of guanidine hydrochloride by the protein and the demonstration, using the well characterized protein, ribonuclease, that valid molecular weights can be obtained in this solvent system without difficulty.

In addition, we have reinvestigated the determination of the molecular weight of native myosin in 0.5 *M* KCl solution by the Archibald technique. From these measurements, the assessment of the subunit weight, and a consideration of the geometry of the native molecule as well as its known X-ray diffraction and optical rotation characteristics, we have arrived at what we consider to be a reasonable model for the myosin molecule.

MATERIALS AND METHODS

Myosin was prepared by a method used previously[7] with the exception that treatment with the Waring blendor was eliminated. The water used for all purposes in the preparation was redistilled from glass and the temperature during all manipulation, after excision of the muscle, was maintained at about 4°.

Protein concentration of these solutions, after centrifugation, was determined either by micro-Kjeldahl analyses or by spectrophotometry using[8] a $D_{280}^{1\%}$ of 6.47. An estimation of the –SH content was immediately made, using the spectrophotometric procedure of BOYER[9]. The ten preparations used in the various phases of this work had an average sulfhydryl content of 8.59 ± 0.08 moles/10^5 g.

For the studies in aqueous guanidine·HCl solution, the myosin was treated with a ten fold excess of N-ethyl maleimide (NEM) to eliminate the possibility of disulfide bridge formation. This mixture was allowed to stand in the cold overnight. Analysis for SH groups the following day were consistently negative, but to insure complete reaction of any small fraction of SH groups that might have escaped detection, the solution was made approximately 5 *M* with respect to guanidine·HCl an hour or two before starting dialysis against 5 *M* guanidine·HCl.

The dialysis was usually carried out at room temperature against four or five volumes of the solvent in a closed container mounted on a magnetic stirrer. The solvent was changed two or three times over a period of a week. Variations in this routine are indicated for the individual experiments.

Extinction coefficient of myosin in 5 M *guanidine·HCl*

The obvious difficulties in the determination of myosin concentration in 5 *M* guanidine·HCl solutions led to our use of spectrophotometry for this purpose. In a preliminary report[10], the values for the partial specific volume, $\bar{V}$, presented were based on the $D_{280}^{1\%}$ determined for myosin in 0.5 *M* KCl. To obtain standards for the extinction coefficient in 5 *M* guanidine·HCl, myosin treated with NEM was dialyzed exhaustively in the cold against dilute NH_4OH (pH 10–10.5). This material was then lyophilized and dried over P_2O_5 *in vacuo* at room temperature for several days. Samples of the dried material were dissolved in 5.0 *M* guanidine·HCl or 5.0 *M* guanidine·HCl–0.05 *M* PO_4, pH 7.5. Dry weights on the P_2O_5 dried material (dry weight = 95.74 %) were obtained on samples dried over P_2O_5 at 100° *in vacuo* for 24 h.

The difference spectrum of solution *vs.* solvent was determined in a Cary spectrophotometer. At the wave length maximum of 276 mμ, the average value for the O.D. of a 1 % solution was $D_{276}^{1\%} = 5.07$. It is of interest that the $D_{275-280}^{1\%} = 4.90$ as calculated from the amino acid composition data of KOMINZ *et al.*[11]. MIHALYI's[12] value for the O.D. of myosin at a wavelength of 280 mμ in 0.5 M KCl, and corrected for turbidity is $D_{280}^{1\%} = 5.88$. The comparable value for myosin in 5 M guanidine·HCl at 280 mμ is $D_{280}^{1\%} = 4.83$. This indicates that the extinction coefficient of myosin in 0.5 M KCl is about 22% higher than that of myosin in 5 M guanidine·HCl and suggests, as in the case of a number of other proteins[13, 14], a decrease in the extinction coefficient of some of the tyrosine residues on unfolding the hydrogen bonded secondary and tertiary structure.

Density determinations

All densities were determined using a 25-ml Leach pycnometer equilibrated in a constant temperature bath maintained at 20.45° ± 0.01°. The bath temperature was established by means of a National Bureau of Standards calibrated thermometer. At least two determinations of the density of each solution were made and the apparent specific volume ($\bar{V}$) was calculated from the relationship

$$\bar{V} = \frac{1}{\rho_0} - \frac{1}{c}\left(\frac{\rho - \rho_0}{\rho_0}\right) \tag{1}$$

where ρ_0 and ρ are densities of solvent and solution respectively, and c is the concentration in g/ml.

Ultracentrifugation

Molecular weight determinations by the Archibald approach to sedimentation equilibrium method and using the schlieren optical system were conducted as outlined by SCHACHMAN[15] employing only the meniscus position and the corresponding equation

$$\mathrm{M} = \frac{RT}{(1 - \bar{V}\rho)\omega^2} \cdot \frac{(\mathrm{d}c/\mathrm{d}x)m}{x_m c_m} \tag{2}$$

and the equation of KLAINER AND KEGELES[16] for correction of concentration (at the meniscus)

$$c_m = c_0 - \frac{1}{{x_m}^2} \int_{x_m}^{X} x^2 (\mathrm{d}c/\mathrm{d}x)\mathrm{d}x \tag{3}$$

where M, R, T, $\bar{V}$ and ρ have their usual designations, ω is the angular velocity, c_m and $(\mathrm{d}c/\mathrm{d}x)_m$ are the concentration and concentration gradient at the position x_m, which is the distance from the axis of rotation to the meniscus and X refers to a position in the center of the cell where $\mathrm{d}c/\mathrm{d}x = 0$; c_0 is the initial concentration of protein in the cell.

The necessary measurements of the photographic plates were made with a Bausch and Lomb microcomparator (Bench Comparator 33-12-11) equipped with a 50 × objective and a special rotating stage for alignment of the plate. Horizontal alignment was insured using the image of the bar across the dummy cell band. The edges of the dummy cell bands and edges of the meniscus were used for vertical alignment. The center line of the meniscus band was established by measurement of the band edge at positions above and below the bar image. Use of the flanking fringes of the meniscus band as recommended by TRAUTMAN[17] was not attempted, since these fringes were not well delineated in our optical system. Measurements were started approximately in the center of the cell, five or six points being employed to establish the plateau region. At the boundary position thirty to forty readings were taken, with 0.05 mm intervals over the linear portion of the schlieren pattern. These readings were then plotted on 40 × 50 cm graph paper on a scale of 1 mm = 0.5 μ. The best line was then drawn through the points, extrapolating to the center of the meniscus. Values were taken from the line at suitable intervals to give 20 to 30 points for subsequent calculation. In the early stages of the study, solution and solvent runs were made in the cell without taking it apart. No appreciable salt distribution was detected at any of the speeds employed.

The initial concentration, c_0, was obtained from a separate ultracentrifuge run using a standard 12 mm, 4° sector synthetic boundary cell[18]. The centerpiece of the cell was coated with a thin film of an acrylic resin. A commercial aerosol preparation was used for this purpose. All Archibald as well as velocity sedimentation runs were made using a Kel-F centerpiece.

When the molecular weight of ribonuclease in 5 *M* guanidine·HCl was determined, speeds of 12,000 to 15,000 rev./min were employed; for the studies on myosin in the same solvent, velocities of 9000–12,000 rev./min were used. In both cases the temperature was maintained at or near 20° with the RTIC unit supplied with the instrument. For native myosin in 0.5 *M* KCl, the speed used was 5227 rev./min and the temperature was maintained at 4–6°. The RNase runs were of relatively short duration, 2–4 h; for myosin in either solvent the runs were 4–26 h. All velocity sedimentation experiments using 5 *M* guanidine·HCl as solvent were conducted at 59,780 rev./min at or near 20° and sedimentation coefficients corrected to a standard state of water at 20° using the viscosity and density of the guanidine·HCl solvent.

A Spinco model H electrophoresis apparatus was utilized for the diffusion experiments. Diffusion was followed by means of the interferometric optical system and diffusion coefficients determined according to the LONGSWORTH[15] method of analysis of the Rayleigh interference patterns. Because of the high index of refraction of aqueous solutions of 5 *M* guanidine·HCl as compared to the bath liquid of the instrument, it was necessary to increase the path length of the "reference" beam in order to bring the fringe system into view. This was accomplished by inserting an optically flat glass plate (American Instruments Co., Silver Spring, Md.) of the proper thickness. This compensation was not an ideal solution to the problem, since the intensities of the two beams were not the same and the fringe system was, therefore, not quite as sharp as in runs made using dilute, aqueous, salt solvents. All diffusion experiments were conducted at 10°; correction to the standard state of water at 20° involved the determination of the viscosity and density of the solvent at the two temperatures.

The Bausch and Lomb microcomparator was used in measuring the fringe patterns from the photographic plates used for the diffusion experiments.

Viscosity

A 1-ml Ostwald type viscometer was used for all viscosity measurements. The capillary tubing of the viscometer was approximately 90 cm long and 0.06 cm inside diameter. Average shear gradient was 300 sec^{-1}. Outflow time for water at 25° was 70 sec.

Optical rotation

Optical rotation and rotatory dispersion measurements were obtained through the use of a Rudolph model 80 precision photoelectric polarimeter equipped with an oscillating polarizer prism. Water from a large volume, external water bath was circulated through jacketed polarimeter tubes to insure temperature control.

RESULTS

The molecular weight of myosin

In the course of our studies on the substructure of myosin we were led to re-examine the present evidence for the molecular weight of the native protein, since an analysis of the assembly pattern of the subunits requires precise information about the size and shape of the parent molecule.

Much of the variation in the reported values for the apparent molecular weight of myosin, which has ranged all the way from 4.2 to $8.5 \cdot 10^5$ g/mole, can be attributed to a marked tendency for the myosin monomers to aggregate to higher molecular weight polymers. This process is clearly evident from the work of LAKI AND CARROLL[19], who demonstrated that the sedimentation patterns of freshly prepared myosin undergo distinct changes, indicative of aggregation, when this protein stands at room temperature for only a few hours. On the other hand, at much lower temperatures (less than 5°) the sedimentation and viscosity properties of myosin remain unchanged for several days[20].

Most of the reported molecular weights for myosin range between values of 500,000 and 650,000, but quite recently two separate groups, using the precise, approach-to-equilibrium technique of Archibald, have obtained values as low as 420,000. In the study of VON HIPPEL *et al.*[21], the molecular weight was determined at a single concentration (0.5 %) using conventional Schlieren optics. These workers pointed out that no significant concentration dependence should be expected for myosin, since the second virial coefficient of this substance (in 0.5 M KCl) was shown to be essentially zero as determined from light scattering studies[22-24]. MOMMAERTS AND ALDRICH[25] also used the Archibald method in their study, but the mass distribution near the meniscus was obtained from the Rayleigh interference optical system. Measurements were made at three concentrations: 5, 3.8 and 2.5 mg/ml. Since the Rayleigh system has not been standardized as yet for Archibald studies using proteins of known molecular weight, its use for myosin must be considered preliminary (see SCHACHMAN[26] for a discussion of this point).

It is surprising that a particle with the known asymmetry and high molecular weight of myosin should exhibit negligible interaction in the concentration range employed for equilibrium sedimentation studies. In fact the osmotic pressure data of PORTZEHL[27], obtained on myosin preparations at 1°, yield a second virial coefficient, A_2, of about

$$0.90 \cdot 10^{-4} \frac{\text{moles-ml}^*}{\text{g}^2}$$

As a result of this discrepancy a number of Archibald studies were made on fresh myosin preparations at concentrations of protein spanning the range 1.35 to 6.0 mg/ml. Two different myosin preparations were employed in this investigation.

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* Utilizing the equation $1/RT\ (\pi/c) = 1/M_n + A_2c$ where π is the osmotic pressure, c the concentration, M_n, the number average molecular weight and A_2, the second virial coefficient.

Solutions were all made up in the cold (4°). The rotor was also precooled and at no time either prior to or during the Archibald run did the temperature of any of the solutions rise above 6°. In the calculation of molecular weight, a number of frames taken at different times were analyzed and averaged for some of the runs made on sample No. 1 and for each of the runs made on sample No. 2. Results are presented in Fig. 1, where a plot of the reciprocal of apparent molecular weight, $1/M_{app}$, versus concentration, is linear over the concentration range examined. The data show a decided dependence of molecular weight on concentration and have been fitted by the method of least squares giving the equation:

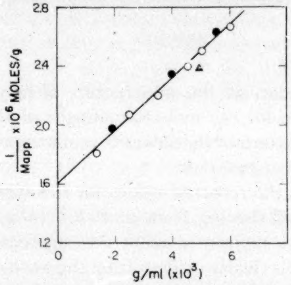

Fig. 1. Concentration dependence of the reciprocal of the apparent molecular weight, $1/M_{app}$, of myosin in 0.5 *M* KCl. ○, sample No. 1; ●, sample No. 2; ▲ $1/M_{app}$ taken from von Hippel *et al.*[21].

$$\frac{1}{M_{app}} = 1{,}616\cdot 10^{-6} + 1{,}793\cdot 10^{-4}c \tag{4}$$

where c is given in g/ml. At infinite dilution the value of the molecular weight is 619,000.

The second virial coefficient may be readily derived (*cf.* Wales[28]) from the approach-to-equilibrium data. In an Archibald run, equilibrium is maintained at all times at the meniscus (or bottom of the cell) during the run. Assuming unit cross section in the ultracentrifuge cell, the change in potential energy in moving c grams of material through the distance $\mathrm{d}x$ to a plane where the concentration is $c + \mathrm{d}c$ will be balanced by the work done against the osmotic pressure: $(\delta\pi/\delta x)\,\mathrm{d}x$. At the meniscus the condition for equilibrium prevails.

$$c(1 - \overline{V}\rho)\omega^2 x\,\mathrm{d}x = \frac{\delta\pi}{\delta x}\,\mathrm{d}x \tag{5}$$

The osmotic pressure of a solution may be expressed in terms of concentration c:

$$\pi = RT\left(\frac{1}{M}c + A_2c^2 + A_3c^3 + \ldots\right) \tag{6}$$

where A_2 is defined as the 2nd virial coefficient. Since

$$\left(\frac{\delta\pi}{\delta x}\right)_{T,P} = \left(\frac{\delta\pi}{\delta c}\right)_{T,P}\frac{\mathrm{d}c}{\mathrm{d}x} \tag{7}$$

it follows that

$$\frac{\delta\pi}{\delta x} = RT\left(\frac{1}{\mathrm{M}} + 2A_2c + 3A_2c^2 + \ldots\right) \tag{8}$$

Dropping terms of higher power in c and substituting in equation (5)

$$\frac{c\omega^2 x(1 - \bar{V}\rho)}{RT\cdot\frac{\mathrm{d}c}{\mathrm{d}x}} = \frac{1}{\mathrm{M}} + 2A_2c \tag{9}$$

This equation is therefore of the form of equation (4) above, with the slope of equation (4) equal to twice the second virial coefficient. From equation (4), $A_2 = 0.897\cdot 10^{-4}\,\frac{\text{moles-ml}}{\text{g}^2}$ for myosin in 0.5 M KCl, in very good agreement with PORTZEHL's value.

From purely theoretical considerations, ZIMM[29] has evaluated the second virial coefficient for long rods of uniform thickness in which l, the length of the rod, is much greater than the diameter, d. He finds

$$A_2 = \frac{\pi N_0 d l^2}{4\mathrm{M}^2} \tag{10}$$

where M is the molecular weight and N_0, Avogadro's number. The best evidence to date indicates that the myosin molecule behaves as a rigid rod in solution[23, 24] (see DISCUSSION) and it is of interest to calculate the second virial coefficient from the molecular weight determined above and the best dimensions for length and diameter. We assume l = 1650 Å and d = 25 Å (see DISCUSSION). The calculation gives

$$A_2 = 0.83\cdot 10^{-4}\,\frac{\text{moles-ml}}{\text{g}^2}$$

in good agreement with the value calculated from the Archibald studies and demonstrating again that an appreciable concentration dependence of molecular weight on concentration should be expected for the myosin molecule.

We may also note that in the approach to equilibrium experiment, the net transport of mass across the meniscus, x_m, and the bottom of the cell, x_b, is zero so that

$$c_m S\omega^2 x_m = D\left(\frac{\delta c}{\delta x}\right)_m \quad \text{or} \quad \frac{\left(\frac{\delta c}{\delta x}\right)_m}{x_m c_m} = \omega^2\frac{S}{D} \tag{11}$$

If the angular velocity of the rotor is held constant for two runs of the same homogeneous protein at concentrations c_1 and c_2, then from equations (2) and (11),

$$\mathrm{M}_1 = K\frac{S_1}{D_1} \quad \text{and} \quad \mathrm{M}_2 = K\frac{S_2}{D_2} \quad \text{or} \quad \frac{\mathrm{M}_1}{\mathrm{M}_2} = \frac{S_1D_2}{S_2D_1} \tag{12}$$

where the subscripts 1 and 2 refer to concentrations c_1 and c_2, respectively.

The diffusion coefficient of myosin has been determined by PARRISH AND MOMMAERTS at 4° and is only slightly dependent on concentration. Let $c_1 = 0$ and $c_2 =$ 0.5 %. Then $D_2/D_1 = 0.94$ according to PARRISH AND MOMMAERTS[30]. The ratio of sedimentation coefficients for myosin in 0.5 M KCl for these two concentrations may be obtained from the data of HOLTZER AND LOWEY[24], PARRISH AND MOMMAERTS and VON HIPPEL *et al.*[21], giving as an average $S_1/S_2 = 1.57$. Therefore $\mathrm{M}_1/\mathrm{M}_2 = 1.48$, or the observed molecular weight would be expected to increase about 50% in Archibald studies over this concentration interval. This compares favorably with the 55% increase observed in Fig. 1 and yields an approximate second virial coefficient,

$$A_2 = 0.77 \cdot 10^{-4} \frac{\text{moles-ml}}{\text{g}^2}$$

We are unable to account for the difference between the value of the second virial coefficient calculated in the present study and that obtained from light scattering measurements. It should be noted, however, that light scattering investigations normally involve a concentration range of about 0.5 mg/ml. The total molecular weight change expected in this range should be only about 3%, judging from Fig. 1, and may well be within the error of the light scattering measurements. It may be inquired as to why recent light scattering studies give molecular weights as low as 493,000[24]. The value of the molecular weight from light scattering studies is markedly sensitive to the refractive index increment since the value of d*n*/d*c* appears as a square term in the Rayleigh equation. The refractive index increment for myosin has had a remarkably unstable existence in the literature[23, 24, 31].

An important feature of the Archibald method is the ease with which inhomogeneity can be detected. In solutions of myosin the presence of a significant amount of aggregation should be revealed by a lowering in the apparent molecular weight as centrifugation proceeds. Molecular weights determined at various times during the centrifugation on three different concentrations of myosin are summarized in Table I. The invariance of the apparent molecular weight with time supports our

TABLE I

THE MOLECULAR WEIGHT OF MYOSIN IN 0.5 *M* KCl VERSUS TIME OF CENTRIFUGATION (ARCHIBALD METHOD)

Concentration g/ml ($\times 10^3$)	*Time (min)*	*Apparent mol. wt. ($\times 10^{-5}$)*
1.90	74	5.13
	138	4.85
	202	5.46
	330	4.86
	458	5.11
	586	4.92
	714	5.21
	842	4.90
		Average = 5.06
4.00	130	4.22
	386	4.32
	642	4.24
		Average = 4.26
5.53	129	3.71
	321	3.81
	513	3.86
		Average = 3.80

view that the myosin preparations used in the present study are devoid of any significant amount of aggregated material*.

In Table II we have assembled molecular weight data for myosin obtained by a number of other workers and based on velocity sedimentation, diffusion, viscosity and light scattering studies. In all of the studies which are quoted, precautions were taken to avoid aggregation. The average of these studies gives $M = 6.03 \cdot 10^5$. If the present study is included, the average of all studies is $6.06 \cdot 10^5$. It is our feeling, therefore, that the weight of evidence places the molecular weight of myosin very close to 600,000 and we shall assume in all of our calculations that the true weight is $6.19 \cdot 10^5$ g/mole.

TABLE II

A SUMMARY OF MOLECULAR WEIGHT DATA ON MYOSIN OBTAINED BY VARIOUS PHYSICAL METHODS

Method	*Parameters used in calculations**	*References*	*Molecular weight* ($\times 10^{-5}$)
S and D	$S_{20}^{\mathrm{w}} = 6.50 \cdot 10^{-13}$	Average of 21, 30 and 24	5.53
	$D_{20,\mathrm{w}} = 1.05 \cdot 10^{-7}$	30	
Light scattering	$dn/dc = 0.208$ ml/g	24	4.93
	$dn/dc = 0.191$ ml/g	23	6.50
S and $[\eta]$	$S_{20}^{\mathrm{w}} = 6.50 \cdot 10^{-13}$	Average of 21, 30 and 24	7.16
	$[\eta] = 2.17$ dl/g	24	

* a $\bar{V}$ of 0.728 ml/g has been assumed in all calculations[30].

Physical chemical properties of myosin in 5 M *guanidine·HCl*

Optical rotatory properties: Before proceeding to an examination of the solution properties of myosin in 5 *M* guanidine·HCl, it is important to determine the degree of unfolding of the polypeptide chains in this solvent. We have thought that, if the hydrogen bonded secondary structure of the myosin molecule could be eliminated, the packing forces of individual chains would be insufficient to hold the chains together and essentially complete dispersion would result. The specific levo-rotation of myosin increases from $[\alpha]_D^{20} = -27.0°$ to $[\alpha]_D^{20} = -90.0°$ (corrected for index of refraction of solvent) on transferring the protein from 0.5 *M* KCl to 5 *M* guanidine·HCl. This change is complete in the time required for measurement. Moreover, the value of the Drude parameter, obtained from a plot of $\lambda^2[\alpha]$ *versus* $[\alpha]$, decreases under these conditions from $\lambda_c = 290$ mμ to $\lambda_c = 218$ mμ. These changes are characteristic of the variations expected in these parameters on unfolding a tightly-coiled hydrogen bonded polypeptide chain[32] and suggest that little, if any, secondary structure remains in the presence of this solvent. Further evidence to support this position is obtained from a plot of $[\alpha]_t$ versus temperature (see Fig. 2), which exhibits a negative temperature coefficient over the entire range examined. Such behavior is expected for unfolded, optically asymmetric chains as predicted by the rule of KAUZMANN AND EYRING[33, 34].

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* Although M_{app} is concentration dependent, the change in protein concentration at the meniscus, c_m, over the time intervals employed in the present measurements, was too small to produce a detectable trend in the molecular weight.

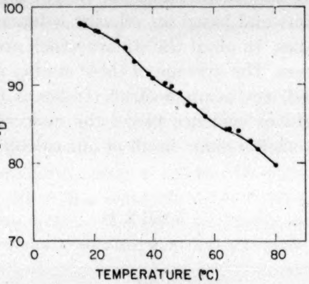

Fig. 2. Effect of temperature on the specific levo-rotation of myosin ($[\alpha]_D$) in 5 *M* guanidine·HCl.

Partial specific volume determinations: Molecular weight determinations by the Archibald method requires a knowledge of the partial specific volume of the protein under the solvent conditions employed. The values obtained for the partial specific volume of myosin and ribonuclease in 5 *M* guanidine hydrochloride are presented in Table III. All the values presented represent the average of duplicate density determinations. The values for both proteins are about 1 % lower than the partial specific volumes in water (0.728 and 0.695 ml/g respectively).

TABLE III

THE PARTIAL SPECIFIC VOLUMES ($\bar{V}$) OF MYOSIN AND RIBONUCLEASE IN 5 *M* GUANIDINE·HCl*

	Preparation	*Concentration (g/ml × 10³)*	$\bar{V}$ *ml/g*
Myosin	2–9	15.0	0.708
	5–13	26.7	0.731
		22.3	0.728
		16.4	0.723
		12.7	0.724
	6–15	20.6	0.721
		10.2	0.708
		Average =	0.720
Ribonuclease		24.0	0.685

* Temperature, 20.23°.

Determination of the molecular weight of myosin in 5 M *guanidine·HCl by the Archibald method*: As stated earlier, ribonuclease was selected as a well defined substance for preliminary experiments to determine the reliability of molecular weight determinations in concentrated aqueous solution of guanidine·HCl by the Archibald equilibrium method. The average molecular weight at two different protein concentrations (1.3 and 2.0 %), was 13,000 ± 500. We note that TRAUTMAN AND CRAMPTON[35] have recently obtained an apparent molecular weight of 12,500 for this protein in 6 *M* urea using the Archibald method.

Turning now to myosin, it was evident quite early from our velocity sedimentation experiments that myosin is dissociated into smaller molecular weight units by concentrated guanidine·HCl and, as will be discussed below, both sedimentation and diffusion studies suggested that these smaller units might be homogeneous with respect to size. Nevertheless, our first concern was a test of homogeneity of our material in the approach to equilibrium technique. If units of significantly different mass were present, one would expect a change in apparent molecular weight with time of centrifugation. Table IV presents a summary of molecular weight results on two different preparations for time intervals up to 26 h. The apparent molecular weight remained essentially constant with time and it appears likely that the destruction of the secondary, hydrogen bonded structure of myosin in 5 *M* guanidine·HCl gives rise to polypeptide chains of closely similar, if not identical mass.

TABLE IV

THE MOLECULAR WEIGHT OF MYOSIN IN 5 *M* GUANIDINE·HCl VERSUS TIME OF CENTRIFUGATION (ARCHIBALD METHOD)

4.88 mg protein/ml		*8.87 mg protein/ml*	
Time (min)	*Apparent mol. wt.* $\times 10^{-5}$	*Time (min)*	*Apparent mol. wt.* $\times 10^{-5}$
33	0.97	29	0.65
97	0.96	61	0.80
161	0.88	93	0.74
225	1.17	125	0.79
315	1.07	157	0.82
443	1.26	189	0.78
571	1.24	221	0.79
699	1.00	285	0.78
		349	0.74
Average =	1.07	733	0.67
		861	0.67
		1400	0.75
		1530	0.73
		Average =	0.75

The reciprocal of apparent molecular weight of myosin in 5 *M* guanidine·HCl is plotted as a function of concentration in Fig. 3. The points in the figure represent average values obtained by analysis at several time intervals and also represent the combined results obtained with four different myosin preparations. The line, obtained by the method of least squares (weighting the points with respect to the number of determinations involved) corresponds to

$$\frac{1}{\mathrm{M_{app}}} = 0.457 \cdot 10^{-5} + 9.897 \cdot 10^{-4} c \tag{13}$$

giving a molecular weight at infinite dilution, $\mathrm{M} = 2.19 \cdot 10^5$ g/mole and providing as accessory information the second virial coefficient

$$A_2 = 4.95 \cdot 10^{-4} \frac{\text{moles-ml}}{\mathrm{g}^2}$$

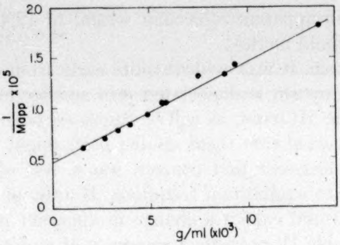

Fig. 3. Concentration dependence of the apparent molecular weight, $\frac{1}{M_{app}}$, of myosin in 5 M guanidine·HCl.

Diffusion studies on myosin in 5 M *guanidine·HCl*: Measurements of diffusion coefficients of myosin at differing concentrations in 5 M guanidine·HCl are summarized in Table V. One diffusion analysis was also determined from the boundary spreading in a synthetic boundary cell run. This diffusion coefficient was corrected for the dependence of sedimentation coefficient on concentration according to the theory of FUJITA[36] as employed by BALDWIN[37]. At a concentration of 0.67 %, a value of $D_{20}^{w} = 2.0 \cdot 10^{-7}$ cm²/sec was obtained.

TABLE V

DIFFUSION OF MYOSIN IN 5 M GUANIDINE·HCl

Concentration *g/ml* (× 10^3)	$D_{20,w}$ × 10^7
1.61	2.03
2.10	2.05
2.68	1.93
3.51	1.94

Recalling the discussion on the relationship of M_{app}, S and D given in section II, a comparison of M_{app} and S_{20}^{w} for 0 and 0.5 % protein leads us to expect a change of approximately 5 % in D within this concentration range and actually indicates that D should increase with increasing concentration.

Typical data obtained from the interference patterns of a single diffusion run (concentration = 2.68 mg/ml; 17 fringe pairs) gave $\Delta H/\Delta h\ (4Dt)^{1/2}$ equal to 0.1628 ± 0.0047. For a true Gaussian distribution, the values of $\Delta H/\Delta h\ (4Dt)^{1/2}$ should be invarient throughout all fringe pairs. The difficulties presented by the presence of the high concentration of salt, as well as the great asymmetry in the macromolecule, introduced more uncertainty into the diffusion coefficient than is normally expected.

Velocity sedimentation of myosin in 5 M *guanidine·HCl*

The velocity sedimentation properties of myosin in 5 M guanidine·HCl have been investigated with a view toward obtaining molecular weight data to supplement the Archibald studies in this solvent. Moreover, it was of interest to examine the sedimentation patterns for evidence of gross heterogeneity. Most of the myosin solutions were examined in a conventional 12 mm, 4° sector cell, but several ultracentrifuge runs were also made using the synthetic boundary cell. The purpose of these latter studies was the detection of any low molecular weight material which might be released during the unfolding of the myosin molecule in guanidine·HCl. Such material, if present in significant amount, would form a concentration gradient at the solvent–solution interface of the synthetic boundary cell and would become apparent as a Schlieren peak when the larger molecular weight subunits sedimented away from the interface. In fact no significant amount of low weight material was observed in these studies. Only a single, relatively rapidly sedimenting boundary (considerably sharpened at the higher concentrations) was apparent at all of the protein concentrations examined (1–12.1 mg/ml) and we may conclude that as far as can be ascertained from these studies, all of the material formed from the interaction of myosin with guanidine·HCl sediments as a single weight class.

A plot of $1/S^{w}_{20}$ versus concentration (g/ml) is quite linear over the concentration range examined (see Fig. 4) and yields by least squares analysis the equation

$$\frac{1}{S^{w}_{20}} = 0.2374 + 38.4\,c;\ S^{w}_{20} = 4.21\ \mathrm{S} \qquad (14)$$

The linearity of the reciprocal sedimentation coefficient versus concentration plot is characteristic of flexible chain polymers, and is consistent with the view that the myosin subunits approach random chains in 5 *M* guanidine·HCl.

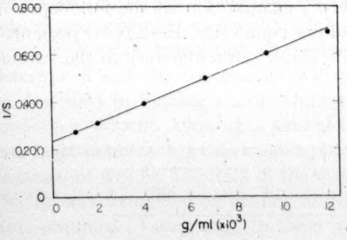

Fig. 4. Concentration dependence of the reciprocal of the sedimentation coefficient, $1/S^{w}_{20}$, of myosin in 5 *M* guanidine HCl.

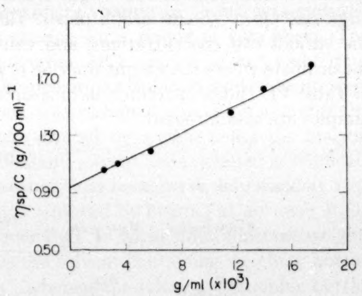

Fig. 5. Reduced viscosity of myosin in 5 *M* guanidine·HCl, versus concentration.

Viscosity of myosin in 5 M *guanidine·HCl*

The reduced viscosity of myosin in 5 *M* guanidine·HCl as a function of concentration is presented in Fig. 5. A stock solution for these studies was prepared by dialyzing a 1.5 % myosin solution against the solvent for several days. The concentration of the stock solution was obtained from the O.D. at 276 mμ as given in MATERIALS AND METHODS. Other concentrations were obtained by dilution of the stock solution with the solvent used for dialysis. The least squares line of Fig. 5 is given by

$$\eta_{sp/c} = 0.9258 + 48.8\,c;\ [\eta] = 0.926\ dl/g \qquad (15)$$

Binding of guanidine·HCl

In the molecular weight determination of myosin in the presence of guanidine·HCl no consideration has been given to the possibility of preferential binding of salt to the protein. If such preferential binding did occur, we would expect both the partial specific volume and the molecular weight of the protein to be affected.

Let us assume that X g of guanidine·HCl are bound per gram protein in these solutions. Then the molecular weight of the complex would be $M_c = M_p\ (1 + X)$, where M_p is the true molecular weight of the protein. Moreover, the partial specific volume of the complex $\bar{V}_c$, can be closely approximated by the sum of the volumes of the protein and guanidine·HCl[39, 40].

$$\bar{V}_c = \frac{X\bar{V}_g + \bar{V}_{p,d}}{X + 1} \tag{16}$$

where $\bar{V}_g$ is the partial specific volume of guanidine·HCl at the concentration employed and $\bar{V}_{d,p}$ is the partial specific volume of the unfolded or denatured protein in the absence of guanidine·HCl.

To estimate the effect of bound guanidine on M_p and on $\bar{V}_p$, the partial specific volume of the native protein, the partial specific volumes of guanidine·HCl at various concentrations of this salt have been determined.

A series of aqueous solutions of guanidine·HCl were made up by weight from Eastman reagent grade material which had been recrystallized from methanol and dried *in vacuo* over P_2O_5 for several days. After determination of densities, a plot of specific volume ($1/\rho$) *versus* weight fraction of guanidine·HCl was constructed on a large size (40 × 50 cm) graph paper. Tangents to the curve were drawn by eye at the various salt concentrations, and values of $\bar{V}_g$ estimated from the intercepts on the ordinate where the weight fraction of guanidine equals one. Results are presented in Table VI. Index of refraction measurements (Abbe refractometer) on the various samples are also included.

TABLE VI

SUMMARY OF DATA ON DENSITY AND INDEX OF REFRACTION OF GUANIDINE·HCl

Weight fraction	*Molar concn.*	*Density (20.15°)*	$\bar{V}$	$n_D^{23.5°}$
0.0	0.0		0.700	1.3327
0.09866	1.060	1.02690	0.731	1.3507
0.18661	2.055	1.05186	0.743	1.3672
0.25649	2.878	1.07184	0.749	1.3809
0.36223	4.177	1.10173	0.754	1.4020
0.44181	5.208	1.12617	0.758	1.4188
0.52271	6.296	1.15074	0.760	1.4362

At a concentration of 5 M guanidine·HCl, $\bar{V}_g = 0.757$ (ml/g). Since the partial specific volume of both myosin and ribonuclease decreases about 1 % on transferring the native protein into 5 M guanidine·HCl, it is clear that the partial specific volume of the unfolded polypeptide chains, apart from guanidine binding, must be lower than that of the native molecule. This observation is consistent with the calculation of LINDERSTRØM-LANG[41] demonstrating a decrease of about 700 ml/mole when the β-lactoglobulin molecule is unfolded. Preliminary measurements on $\bar{V}$ of ribonuclease after unfolding this molecule, through oxidation of the S–S bridges, also indicate[14] that $\bar{V}$ decreases, in this case from 0.695 to 0.682 ml/g.

SCHELLMAN[42] has recently estimated the volume change on rupturing a peptide hydrogen bond from thermodynamic data on the association of urea in aqueous solution. He infers the volume change on unfolding polypeptide structures to be of the order of 2.5 ml per hydrogen bond. If we assume for purposes of discussion that 80% of the amino acids in the myosin molecule are H-bonded, the value of $\bar{V}$ for the unfolded molecule should be about 0.710 ml/g. Thus the disruption of the secondary hydrogen bonded structure in guanidine·HCl can easily account for the decrease in $\bar{V}$ observed.

Assuming $\bar{V}_{p,d}$ of myosin to be 0.710 and utilizing equation (16) above, we estimate that a preferential binding of about 0.25 g guanidine·HCl/g protein would be necessary under these conditions to raise the value of $\bar{V}_c$ to 0.720 ml/g, the observed value. This calculation, though of interest, must be considered as relatively crude in view of the assumptions made above.

From the preceding discussion it will be appreciated that a precise determination of the preferential binding of guanidine·HCl is required to obtain the true molecular weight of the myosin subunits. This has been accomplished through the use of equilibrium dialysis.

Myosin was isolated at 4° as usual and the NEM treated protein precipitated by dilution of the 0.5 *M* KCl solution with ten volumes of water. Solid guanidine·HCl and water were added to the precipitate to give a resulting solution which was 5 *M* with respect to the salt. This solution (approximate volume = 50 ml) was dialyzed versus 500 ml of 5 *M* guanidine·HCl with stirring for a period of two weeks. The solvent was replaced twice with fresh 5 *M* guanidine·HCl solution (after 3 and 6 days time). Following completion of dialysis, the density of solution and solvent were determined and the concentration of protein was obtained spectrophotometrically as described in Section I. All solutions were diluted by weight using the dialysis medium as diluent. Aliquots of 5 ml of solution and solvent were weighed in triplicate into large diameter (35 ml) weighing bottles which had been previously dried at 105° to constant weight. The bulk of the water was removed by heating at 40° over P_2O_5. Drying was completed by heating at 105° over P_2O_5 *in vacuo* until constant weights were obtained (± 0.02 mg). In these studies the solvent-containing weighing bottles assumed constant weight within 48 h at 105°, whereas the solution-containing bottles required 290 h at this temperature to approach constant weight.

Utilizing the information obtained from the type of experiment described above, we may now proceed to a calculation of the preferentially bound guanidine·HCl. The volume of the protein solution inside the dialysis bag may be expressed in terms of the masses and partial specific volumes of the various components. (Calculation shows the Donnan effect to be negligible at these high salt concentrations.) For a volume of 1 ml,

$$X_{H_2O}\bar{V}_{H_2O} + X_g\bar{V}_g + X_c\bar{V}_c = 1 \qquad (17)$$

where X_{H_2O}, X_g and X_c are the masses of water, guanidine·HCl and protein·guanidine·HCl complex, and $\bar{V}_{H_2O}$, $\bar{V}_g$ and $\bar{V}_c$ are the partial specific volumes of these components, respectively. Similarly, a volume of 1 ml of the dialysate outside the dialysis bag may be expressed as

$$X^{\circ}_{H_2O}\bar{V}_{H_2O} + X^{\circ}_g\bar{V}_g = 1 \qquad (18)$$

on drying these solutions,

$$M^i = X^i{}_g + X_c \tag{19}$$

and

$$M^\circ = X^\circ{}_g \tag{20}$$

where M_i is the dry weight of 1 ml of protein solution and M°, the dry weight of 1 ml of dialysate. The mass of guanidine·HCl per milliliter in unbound form inside the dialysis bag is

$$X^i{}_g = X^\circ{}_g - (\bar{V}_c X_c) X^\circ{}_g \tag{21}$$

$$M^i = M^\circ - (\bar{V}_c X_c) M^\circ + X_c \tag{22}$$

and the weight of the complex per milliliter is

$$X_c = \frac{M^i - M^\circ}{1 - M^\circ_c \, V_c} \tag{23}$$

letting X_p = the mass of protein per milliliter, as determined spectrophotometrically, we find

$$\frac{X_c - X_p}{X_p} \times 100 = \% \text{ guanidine·HCl bound} \tag{24}$$

The results of three separate determinations at different protein concentrations are summarized in Table VII. Although there is some variation among the three determinations, it is probable that the amount of guanidine·HCl bound is of the order of 5 % or less and in the calculation of molecular weight we will take the figure of 5 % as the best value.

TABLE VII

THE BINDING OF GUANIDINE·HCl TO MYOSIN

For explanation of symbols see text.

Experiment	*Xp* (*g/ml* × *10³*)	*Mi* (*g*)	*Mo* (*g*)	*Xc–Xp* (*g/ml* × *10³*)	$\frac{X_c - X_p}{X_p} \times 100$
1	10.17	0.48364	0.47637	0.59	5.5
2	20.57	0.49128	0.47637	1.40	6.4
3	26.70	0.49505	0.47669	0.82	3.0

Before leaving this section it seems pertinent to note that since the binding of guanidine·HCl appears to be of the order of 5 % or less for two markedly dissimilar proteins viz. ribonuclease and myosin, it seems reasonable to assume that only a slight preferential binding of this salt to any polypeptide chain will occur. The methods used for examining the subunit molecular weight of myosin in 5 *M* guanidine·HCl should be applicable to other protein systems as well.

DISCUSSION

We now turn to an analysis of the various pieces of information which have been provided in previous sections of this study and attempt to develop a consistent, three dimensional structure for the myosin molecule. Taking account of the bound guanidine·HCl (5 %), and the 18 NEM groups (weight = 2628 g) per individual chain, the most precise determination of the weight of each polypeptide chain which is liberated on unfolding the parent molecule in 5 *M* guanidine·HCl, comes from the Archibald studies yielding M = 206,000. This value is given strong support by the sedimentation, diffusion and viscosity experiments presented above. From sedimentation and diffusion (using the average value of $D_{20}^{\mathrm{w}} = 1.99 \cdot 10^{-7}$ cm^2/sec (Table V)) we calculate a mol. wt. of 172,000. (This value would be raised to 181,000 if we assume a concentration dependence of *D* as required by equation 12 and the sedimentation coefficient data of Fig. 4.) The mol. wt. may also be estimated from the FLORY-MANDELKERN equation[43]:

$$\beta = \frac{S[\eta]^{1/3} N_0 \eta}{\mathrm{M}^{2/3}(1 - V\rho)}$$

where $[\eta]$ is the intrinsic viscosity, S, the sedimentation coefficient, N_0, Avogadro's number, η, the viscosity of the solvent, and M, the molecular weight. We assume β for the individual chain in 5 *M* guanidine·HCl to be $2.5 \cdot 10^6$, the value accepted for a random chain[43]. The molecular weight deduced from this calculation is M = 197,000.

Accepting the Archibald measurements as providing the best values for the molecular weight of the subunit and the native molecule, we observe that there are 6.19/2.06 = 3.01 individual polypeptide chains per myosin molecule. The precision of the data strongly suggests that three polypeptide chains of equivalent mass are associated to form one molecule of native myosin.

The amino acid composition of rabbit myosin has been reported by KOMINZ *et al.*[11]. They find close to 848 amino acids per 10^5 g of myosin. Thus each polypeptide chain of the molecule consists of about 1747 amino acids.

ASTBURY'S[44,45] classical work on muscle and on films of myosin prepared from muscle extracts has demonstrated that myosin has essentially the same X-ray diffraction pattern as α-keratin* and it is probable that the individual chains of native myosin are folded into the α-helical configuration. Assuming the well-known 1.5 Å distance per residue along the helix axis and 100 % α-helix, the length of each polypeptide chain in myosin would be close to 2620 Å. Since we have 3 chains, and since the myosin molecule is highly asymmetric ($b/a = 70$), it is clear that the three α-helices must be arranged side-by-side in a linearly extended fashion. PAULING AND COREY[46] and CRICK[47] have independently proposed that the 5.1 Å spacing observed in the X-ray diffraction patterns of the *kmef* class of proteins is consistent with a coiling of each α-helix into a super coil. This has been assumed to occur through the formation of multistranded cables or ropes, two, three and seven stranded cables being probable structures, in view of packing requirements. Thus it seems likely that the three myosin α-helices are wound about each other to form a three stranded α-rope.

If we assume a pitch of 62 Å[47] and a radius of the super coil = 6 Å[46], the minimum length of the myosin rod would be 2240 Å. The diameter of a 3 stranded α-rope is close to 22 Å[46]. It is of interest to compare these dimensions with those obtained for the size and shape of the myosin molecule from various physical studies.

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* Dr. D. DAVIES of the National Institute of Mental Health has kindly examined the X-ray diffraction patterns of drawn filaments of our myosin preparations. These were obtained from essentially salt-free myosin gels and exhibited the typical diffraction spacings of the *kmef* class of proteins with no indication of any substantial amounts of any other structural element.

Holtzer and Lowey[20] and Parrish and Mommaerts[30] have demonstrated that the results of their light scattering investigations are consistent with a rod model for myosin. The length of the rod was calculated by these workers in the usual way from the estimated radius of gyration. Both the length and diameter of the rod may also be obtained from a combination of the axial ratio, l/d, derived from the Simha[48] equation or the Kirkwood-Auer[49] equation, and the mass of a uniform cylinder in terms of its dimensions and density:

$$m = \frac{M}{N} = \frac{1}{4}\pi d^2 l p$$

Yet another estimation of these parameters may be made from the equation of Zimm given in a previous section. A summary of the lengths and diameters expected for myosin, using the molecular weight and second virial coefficient obtained in the present study, and assuming a rigid rod of uniform thickness, is presented in Table VIII.

TABLE VIII

LENGTH AND DIAMETER OF THE MYOSIN MOLECULE

Method	*Parameters used in calculation*	*Reference*	*Length*
Length:			
1. Light scattering	Radius of gyration	24	1620 Å
Light scattering	Radius of gyration	23	1600 Å
2. Viscosity*	$[\eta] = 2.17$ dl/g	24	1646 Å
molecular weight	$M = 6.19 \cdot 10^5$		
3. Second virial	$A_2 = 0.893 \cdot 10^{-4}$ moles-ml/g^2		
molecular weight	$M = 6.19 \cdot 10^5$		1713 Å
viscosity*	$[\eta] = 2.17$ dl/g	24	
			Average = 1650 Å
Diameter: 24.3 Å			

Combination of average length and methods (1) and (2) give best diameter

* Axial ratio, $l/d = 68$ from either Simha[48] or Kirkwood-Auer[49] equations and $[\eta]$.

The 24.3-Å value for the diameter of the myosin rod is in good agreement with that expected for a three stranded α-rope. But even the minimum pitch length of 62 Å would give a linear extension of such a rope 600 Å beyond the most probable length of the myosin molecule of 1650 Å. We are thus led to the conclusion that a section of the rope of the order of 600–700 Å is folded back upon itself in some manner*. It seems possible that this folded section of the molecule is the so-called heavy meromyosin fragment, which is liberated on brief tryptic digestion of myosin. The axial ratio of the isolated heavy meromyosin segment has been determined by

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* We must keep in mind that although light scattering studies indicate that the myosin molecule is a rigid rod, it is possible that some flexibility of the rod may account for the apparent shortening, of the contour length.

Szent-Györgyi[52]. The average of his estimates of l/d is 18. The heavy folded section of the molecule suggested above with a length of 600 Å and a diameter of 33 Å also has an axial ratio of 18.

Holtzer and Loewy[50] have recently reported molecular weights on the heavy and light meromyosins isolated from tryptic hydrolysates. From Archibald studies they obtain 324,000 for the heavy meromyosin and 126,000 for the light meromyosin. About 6 % of the mass of myosin is liberated as dialyzable fragments during tryptic digestion. The remainder of the mass is confined to the light and heavy components. Granting this information, we observe that if the molecule contains two light and one heavy components, the molecular weight should be 613,000. This value is within 2 % of the molecular weight of 619,000 which we are accepting in the present study.

Thus it is probable that two light and one heavy fragments are liberated during tryptic digestion of myosin, a proposal which is in good agreement with the mass distribution of these components as reported by **Mihalyi and Szent-Györgyi**[51] and **Szent-Györgyi**[52]. We further note that if 600 Å of the end segment of the three stranded α-rope were to be bent back, close packed, but direction counter to the direction of the α-rope, this folded segment should have a molecular weight equal to $1200/2210 \times 6.19 \times 10^5$ or 332,000 a value which closely approximates the observed molecular weight of the heavy component, as determined by **Holtzer and Lowey**[50]. The length of each light component following the reasoning above would be $(2240 - 1200)/2$ or about 520 Å. So that the expected mass for each light meromyosin should be $520/2240 \times 6.19 \times 10^5 = 144{,}000$. This is somewhat higher than the value of 126,000 obtained by **Holtzer and Lowey**, but we have made no correction as yet for the 6 % mass lost during the formation of these fragments. If this mass, amounting to about 37,000 g/mole of myosin, is distributed equally among the light meromyosins, the mass of these segments in the intact native molecule would be close to 145,000. The numbers suggest, then, that most of the dialyzable hydrolysis products from tryptic digestion of myosin come from the three-stranded rope sections rather than from the folded heavy meromyosin section.

It will be evident from the discussion above that the myosin rod cannot have a uniform diameter. Assuming that the heavy section is a uniform cylinder with the same density as native myosin ($\rho = 1.373$ ml/g) a length of 600 Å and a mass of $3.24 \cdot 10^5$, the diameter of this section is about 33 Å. Using the estimated figure of 22 Å as the diameter of the 3 stranded α cable section, we see that the average diameter of the molecule, $d_{av} = 26$ Å, a value in reasonable agreement with the 24.3 Å figure derived from the data presented in Table VIII.

In considering a model for the myosin molecule we have assumed that the polypeptide chains are perfect α-helices and that the pitch of the super coil for the three-stranded rope is the rather minimal distance of 62 Å. We believe that two small portions of the three-stranded rope section, amounting to about 6 % of the total mass, are probably amorphous, in order to account for the action of the various proteolytic enzymes on myosin[6]. If the polypeptide chains in these regions are completely unfolded, the total length of the rod would increase about 150 Å. The heavy or folded section, on the other hand, contains most of the proline of the molecule. This will partially destroy the helical pattern in this segment and will allow generously for folding of the chains. The pitch of the super coil may be somewhat greater than the value we have used, but the actual length of the rod is not too sensitive to this, since even this tight coiling leads to a decrease of only 15 % in the length of the α-rope.

Taking into consideration all of the information presented in the discussion above, it is our feeling that the simplest model which is consistent with the data is that shown in Fig. 6. The proposed model possesses some interesting possibilities as a structure to which we can begin to relate the properties of myosin.

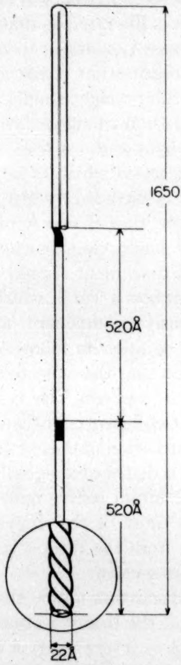

Fig. 6. A proposed model of the myosin molecule.

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THE ENZYMIC DESTRUCTION OF CAROTENOIDS BY ISOLATED CHLOROPLASTS

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SUMMARY

Chloroplasts isolated from sugar beet leaves are shown to contain an enzyme which will oxidize carotenoids. This enzyme is inhibited by cyanide and diethyldithiocarbamate. Carotenoid destruction is also inhibited by a number of antioxidants, and stimulated by the addition of zinc acetate and *o*-phenanthroline to chloroplasts. The enzyme has been extracted from the chloroplasts with sodium deoxycholate and partially purified by fractionation of the extract with ammonium sulphate.

INTRODUCTION

It has been known for some time that destruction of β-carotene can occur in detached leaves, especially after grinding[1]. BOOTH[2] has recently examined various types of plant tissue for the presence of systems which will destroy β-carotene and has found that such destruction only occurs in green tissue. He suggested that the destruction might be enzyme-catalysed. Preliminary experiments in this laboratory[3] have shown that an enzyme system capable of destroying β-carotene is present in chloroplasts isolated from both spinach beet and sugar beet leaves.

In order to investigate the problem further, we have examined the destruction of crocin, a water-soluble carotenoid glycoside, by chloroplasts prepared from sugar beet leaves. The results show that the destruction of crocin, which resembles the destruction of β-carotene, is an aerobic process catalysed by an enzyme which can be extracted from the chloroplasts by treatment with sodium deoxycholate. A preliminary report of these results has already appeared[4].

MATERIALS AND METHODS

Materials

Sugar beet leaves (*Beta vulgaris* var. Sharps Klein Wanzleben E) were harvested from plants grown in a greenhouse, washed in cold water and stored in polythene bags at + 1°C until required. They could be kept for periods of 3–5 weeks without deterioration. Chloroplasts were prepared as previously described[5,6] and resuspended in a solution containing 0.5 M sucrose, 0.067 M phosphate buffer pH 7.3 and 0.01 M KCl.

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Crocin was prepared from commercial saffron. Lipid and some carotenoids were removed by continuous ether extraction, and a stock solution of crocin prepared by extraction of the residue with methanol at room temperature. This solution was kept in the refrigerator. The substrate was prepared by evaporating an aliquot of the methanolic solution to dryness *in vacuo* at room temperature and dissolving the residue in water. The aqueous solution had absorption maxima at 440 and 473 mμ, corresponding with those of pure crocin[7]; a 0.5-ml aliquot diluted to 25 ml with water had an absorbance of 0.385 at 440 mμ in a 1-cm cell. Assuming $E^{1\%}_{1\,\mathrm{cm}} = 1369$ for crocin at 440 mμ (calculated from $E^{1\%}_{1\,\mathrm{cm}} = 3875$ for crocetin dimethyl ester, ISLER *et al.*[8]) the concentration of crocin in the substrate solution is equal to 140 μg/ml.

Lipoxidase, catalase and peroxidase were obtained from L. Light and Co., Colnbrook, Bucks. All other reagents were of the highest grade commercially obtainable. Distilled water, deionised by passing through an Amberlite Monobed MBl resin was used throughout.

Methods

Chloroplast suspension, 0.5 ml crocin solution, and either water or solution of inhibitors to give a final volume of 2 ml were shaken in small conical flasks for 20 min at 30° A 1-ml sample was withdrawn and pipetted into 2 ml acetone in a stoppered tube. 5 ml half-saturated ammonium sulphate solution were added followed by 5 ml *n*-butanol. After shaking and leaving to settle, 5 ml of the upper layer, containing the residual crocin, were transferred to a second tube containing 5 ml water. After addition of 10 ml ether, the tube was stoppered and shaken; the crocin then passed into the lower aqueous layer. The absorbance of this lower layer was measured at 440 mμ.

To compensate for possible non-enzymic oxidation of crocin, boiled chloroplasts were incubated with crocin under identical conditions, and the crocin isolated and estimated as described above. In the majority of cases there was no non-enzymic destruction, and an initial concentration of 70 μg crocin in 2 ml gave, after extraction, a solution which had an absorbance of 0.44 at 440 mμ in a 1-cm cell. A drop in absorbance of 0.1 is thus equal to a loss of 15.9 μg crocin or $15.8 \times 10^{-9}\,M$.

Protein was estimated by the method of LOWRY *et al.*[9].

Extraction of the enzyme is described in the body of the paper. All the extractions and treatments were carried out in a cold room at —1°.

RESULTS

The effect of pH on the reaction was observed by suspending chloroplasts in the usual medium (pH 7.3) and adjusting the pH with either sodium hydroxide or phosphoric acid. As the results (Table I) showed that the pH optimum is between 7.3 and 8.0, all further experiments were carried out at pH 7.3.

Although a stimulatory effect was shown by light (Table II) subsequent experiments were carried out in the dark in which adequate destruction occurred.

The possibility of measuring additional oxygen uptake by chloroplasts in the presence of crocin using conventional manometric techniques was ruled out because an uptake of 1 mole oxygen/mole crocin would only correspond to an oxygen uptake of 1.6 μl. However, when the reaction was carried out in Thunberg tubes which were evacuated at the water pump, 100% inhibition of crocin destruction was found (Table III).

TABLE I

THE EFFECT OF pH ON THE DESTRUCTION OF CROCIN BY INTACT CHLOROPLASTS

Experimental conditions are described in the text. Specific activity is the drop in O.D. per milligram protein/20 min.

pH	*Specific activity*	*Crocin destroyed, as percentage of destruction at pH 7.3*
8.0	0.146	101
7.3	0.145	100
6.5	0.079	53
5.8	0.027	19
4.7	0.004	3

TABLE II

THE EFFECT OF LIGHT ON THE DESTRUCTION OF CROCIN BY INTACT CHLOROPLASTS

The reaction flasks were incubated for the usual time on a flask shaker in a room at 30°; illumination was from a tungsten lamp.

Conditions	*Specific activity*	*Crocin destruction (percentage of dark destruction)*
Dark	0.036	100
Light (1,000 ft. candles)	0.051	142

TABLE III

THE EFFECT OF INHIBITORS ON THE DESTRUCTION OF CROCIN BY INTACT CHLOROPLASTS

Incubation conditions as described in text.

Inhibitor (and concn.)	*% inhibition*
Control	0
Anaerobic conditions	100
KCN $1 \cdot 10^{-4}$ *M*	89
KCN $5 \cdot 10^{-5}$ *M*	63
KCN $1 \cdot 10^{-5}$ *M*	23
DIECA $1 \cdot 10^{-4}$ *M*	59
DIECA $5 \cdot 10^{-5}$ *M*	37
p-Benzoquinone $9.2 \cdot 10^{-4}$ *M*	89
Quinol $1 \cdot 10^{-3}$ *M*	95
Ascorbic acid $1 \cdot 10^{-3}$ *M*	93
Thiourea $1 \cdot 10^{-3}$ *M*	14
Diphenylamine $1.47 \cdot 10^{-5}$ *M*	27
Phenazine methosulphate $1.8 \cdot 10^{-5}$ *M*	69

The inhibitory actions of potassium cyanide and sodium diethyldithiocarbamate were examined at various concentrations and it was found (Table III) that KCN was a more effective inhibitor, giving 63 % inhibition at $5 \cdot 10^{-5}$ *M* compared with only 37 % inhibition by DIECA at the same concentration. It is clear, therefore, that the destruction of crocin requires oxygen and probably involves a metal-enzyme.

The effect of other inhibitors was examined and the results (Table III) showed that hydroquinone and ascorbic acid, which are often used as antioxidants during extraction of β-carotene from leaves, were very effective inhibitors at 10^{-3} *M*. Thiourea at the same concentration was almost ineffective. *p*-Benzoquinone which stimulates the destruction of carotenoids in *Chlorella*[10] here showed a strong inhibitory action. The inhibition of crocin destruction by phenazine methosulphate is similar to that previously found for the destruction of endogenous β-carotene in spinach-beet chloroplasts[3].

o-Phenanthroline alone was found to inhibit crocin destruction (Table IV) while zinc acetate had no effect. However equimolar amounts of the two compounds together greatly stimulated crocin destruction, generally increasing it about 2.5 fold, (Table IV). It may be seen that the stimulatory effect depends to some extent on the ratio of concentration of zinc acetate and *o*-phenanthroline. A similar effect of *o*-phenanthroline and zinc acetate has also been found for the destruction of endogenous β-carotene in spinach-beet chloroplasts[3]. Replacement of zinc by nickel (as nickel sulphate) also stimulated crocin destruction but to a lesser extent; however iron (as ferrous sulphate) merely reversed the inhibitory effect of *o*-phenanthroline.

TABLE IV

THE EFFECT OF Zn ACETATE AND *o*-PHENANTHROLINE ON THE DESTRUCTION OF CROCIN BY INTACT CHLOROPLASTS

Expt.		*Specific activity*	*Crocin destruction as % of control*
1	Control	0.042	100
	Zn acetate $5 \cdot 10^{-4}$ *M*	0.048	113
	o-phenanthroline $5 \cdot 10^{-4}$ *M*	0.027	64
	Zn acetate $5 \cdot 10^{-4}$ *M* *o*-phenanthroline $5 \cdot 10^{-4}$ *M*	0.116	250
	Zn acetate $3.3 \cdot 10^{-4}$ *M* *o*-phenanthroline $5 \cdot 10^{-4}$ *M*	0.116	250
	Zn acetate $1.67 \cdot 10^{-4}$ *M* *o*-phenanthroline $5 \cdot 10^{-4}$ *M*	0.71	167
2	NiSO$_4$ $5 \cdot 10^{-4}$ *M* *o*-phenanthroline $5 \cdot 10^{-4}$ *M*	0.167	117
	Zn acetate $5 \cdot 10^{-4}$ *M* *o*-phenanthroline $5 \cdot 10^{-4}$ *M*	0.185	130
	NiSO$_4$ $5 \cdot 10^{-4}$ *M*	0.143	100
	Control	0.143	100
3	FeSO$_4$ $5 \cdot 10^{-4}$ *M* *o*-phenanthroline $5 \cdot 10^{-4}$ *M*	0.033	100
	Zn acetate $5 \cdot 10^{-4}$ *M* *o*-phenanthroline $5 \cdot 10^{-4}$ *M*	0.109	326
	FeSO$_4$ $5 \cdot 10^{-4}$ *M*	0.024	71
	Control	0.033	100

In an attempt to see whether the enzymic destruction of crocin by chloroplasts might possibly be a coupled oxidation involving lipoxidase or peroxidase, crocin was incubated with these enzymes and their substrates, but without chloroplasts for 20 min at 30°.

In the absence of chloroplasts, lipoxidase and freshly prepared sodium linoleate caused only very slight destruction of crocin (Table V). The possibility of a lipoxidase system being involved in crocin destruction by chloroplasts was further excluded by the experiments in which linoleate was added to chloroplasts incubated with crocin (Table VI). Freshly prepared sodium linoleate actually inhibited crocin destruction, whereas partially peroxidized sodium linoleate stimulated the destruction. Similar effects have been found in the coupled oxidation of β-carotene by metallo-protein systems (J. A. BLAIN, personal communication).

TABLE V

EFFECT OF VARIOUS ENZYMES AND OXIDIZING SYSTEMS ON CROCIN DESTRUCTION IN THE ABSENCE OF CHLOROPLASTS

	Change in O.D. after 20 min
Lipoxidase (4 mg) and sodium linoleate 0.62 mg	0.028
Peroxidase	0.015
Hydrogen peroxide ($5 \cdot 10^{-2}$ *M*)	0.090
Hydrogen peroxide + peroxidase	0.115

TABLE VI

EFFECT OF ADDITION OF VARIOUS ENZYMES AND OXIDIZING SYSTEMS ON CROCIN DESTRUCTION IN THE PRESENCE OF CHLOROPLASTS

Additions to system	*Crocin destruction (percentage of control)*
None (control)	100
Sodium linoleate 0.62 mg/ml	67
Sodium linoleate 0.62 mg/ml (partially oxidized)	137
Hydrogen peroxide $5 \cdot 10^{-2}$ *M*	72
Catalase 0.5 mg/ml + ethanol 2.5 %	109
Catalase 1 mg/ml	87
Catalase 1 mg/ml + ethanol 2.5 %	74

Hydrogen peroxide ($5 \cdot 10^{-2}$ *M*) destroyed an appreciable amount of crocin and this destruction was catalyzed by peroxidase (Table V). If the chloroplasts were oxidizing crocin peroxidatically, addition of hydrogen peroxide to chloroplasts should stimulate crocin destruction and addition of catalase or catalase plus ethanol should inhibit it by removing the hydrogen peroxide. It may be seen (Table VI) that addition of hydrogen peroxide to chloroplasts actually inhibited crocin destruction and that inhibition with catalase and ethanol could only be obtained at very high concentrations of catalase. It seems unlikely, therefore, that chloroplasts destroy crocin by a peroxidatic type of reaction.

Purification of the enzyme

In order to extract the enzyme from intact chloroplasts, they were disrupted by several treatments which included resuspension in distilled water, maceration with acetone at —20° followed by extraction of the residue with distilled water, and maceration with buffered solutions of the detergents digitonin, Tween 60 and Triton-X-100. In none of these cases was any appreciable fraction of the enzyme activity extracted. However, extraction with 1 % sodium deoxycholate (DOC) in phosphate buffer 0.067 *M* pH 7.3 was effective. A convenient procedure was to macerate the chloroplasts in a hand-operated all-glass homogenizer, leave the suspension for 30 min and then centrifuge for 10 min at 3,700 × *g*. 50–100 % of the enzyme activity was found in the supernatant (A_2) which could then be further fractionated with ammonium sulphate either before or after dialysis.

Fractionation of deoxycholate extract before dialysis

Addition of saturated ammonium sulphate solution to extract A_2 so that the final ammonium sulphate concentration was 13 % saturation gave a precipitate (B_1) which was removed by centrifugation for 15 min at 3,700 × *g*.

Further treatment of the supernatant to bring the ammonium sulphate concentration to 28 % saturation gave a second precipitate (C_1) which was also removed by centrifugation.

Both precipitates B_1 and C_1 were dissolved in phosphate buffer, 0.067 *M* pH 7.3; their activity towards crocin and their response to *o*-phenanthroline plus zinc acetate was examined.

It was found (Table VII) that all fractions destroyed crocin, and that *o*-phenanthroline plus zinc acetate stimulated fraction C_1, had little effect on A_2, and inhibited B_1 very strongly.

TABLE VII

ACTIVITY OF FRACTIONS OBTAINED BY AMMONIUM SULPHATE PRECIPITATION OF A 1% SODIUM DEOXYCHOLATE EXTRACT OF CHLOROPLASTS

Dialysis where indicated, was for 3 h against ice-cold tap-water.

	Fraction	*Specific activity*	*Response to added zinc acetate and o-phenanthroline both at $5 \cdot 10^{-4}$ M*
A_2	1 % DOC extract	0.059	+ 6 %
A_3	A_2 dialysed for 3 h	0.091	+ 9 %
B_1	Precipitated between 0–13 % saturation with ammonium sulphate	0.089	Marked inhibition (— 75 %)
B_2	B_1 dialysed for 3 h	0.064	No effect
C_1	Precipitated between 13–28 % saturation with ammonium sulphate	0.118	+ 108 %
C_2	C_1 dialysed for 3 h	0.129	+ 65 %

A_2, B_1 and C_1 were dialysed for 3 h against ice-cold tap water yielding dialysed fractions A_3, B_2 and C_2 respectively. A_3 and C_2 showed a greater crocin destroying activity than the corresponding undialysed fractions, whereas B_2 was less active than B_1. The loss of activity in B_2 is almost certainly due to its inherent instability, since solutions of B_1 lost over 50 % of their activity after 24 h at 0°. The increase in the activity of A_3 and C_2 is probably due to the removal of some of the deoxycholate.

The presence of two enzyme fractions B_1 and C_1 one of which, B_1, is unstable is consistent with the observation that chloroplasts from occasional batches of leaves showed a marked drop in activity on standing at + 1°.

Fractionation of deoxycholate extract after dialysis

A deoxycholate extract of chloroplasts (A_2), prepared as described above, was dialysed for 18 h against ice-cold tap water (A_4); the precipitate which formed (K) was removed by centrifugation and resuspended in phosphate buffer 0.067 *M* pH 7.3.

Fractionation of the supernatant with ammonium sulphate gave precipitates. L and M at 10 % and 20 % saturation respectively which were removed by centrifugation and resuspended in phosphate buffer as before.

All the fractions, when assayed, showed an increase in specific activity compared with that of the complete dialysed extract A_4 (Table VIII).

Fraction M differs from all fractions previously isolated in showing a 435 % stimulation by *o*-phenanthroline plus zinc acetate; there is also indication of another enzyme in fraction L which is showing much less response to *o*-phenanthroline zinc acetate. This may well correspond with fraction B_1 from the previous fractionation (Table VII). Probably this fraction was contaminated with part of (M) which remained unchanged, while the other part consisting of the unstable enzyme had become inactive.

TABLE VIII

ACTIVITY OF FRACTIONS OBTAINED BY AMMONIUM SULPHATE PRECIPITATION OF A DIALYSED 1 % SODIUM DEOXYCHOLATE EXTRACT OF CHLOROPLASTS

The extract was dialysed for 18 h against ice-cold tap-water.

	Fraction	*Specific activity*	*Response to added zinc acetate and o-phenanthroline both at $5 \cdot 10^{-4}$ M*
A_4	Complete dialysed extract	0.03	+ 156 %
K	Precipitate formed after dialysis	0.042	+ 120 %
L	Precipitated between 0–10 % saturation ammonium sulphate	0.127	+ 96 %
M	Precipitated between 10–20 % saturation ammonium sulphate	0.062	+ 435 %

The oxidation of solubilized β-carotene

Solutions of pure crystalline β-carotene in chloroform and of Triton-X-100 also in chloroform were mixed and evaporated to dryness *in vacuo* at room temperature, and the residue shaken with water; solid material was removed by centrifugation. Aliquots of this solution were incubated in the usual manner with fraction M (Table VIII). The carotene was estimated by pipetting a 1-ml aliquot into 2 ml of acetone, adding 3 ml petroleum ether (b.p. 80–100°) and 5 ml half-saturated ammonium sulphate. The absorbance of the carotene in the epiphase was measured at 450 mμ in 5-mm cells. Fraction M destroyed 1.6 μg β-carotene in 30 min and in the presence of *o*-phenanthroline-zinc acetate destroyed 6.4 μg ($3.0 \cdot 10^{-9}$ *M* and $12.0 \cdot 10^{-9}$ *M* β-carotene respectively).

DISCUSSION

It is clear that sugar beet chloroplasts destroy crocin enzymically; the similarity between the enzymic destruction of crocin and that of endogenous β-carotene is shown by the finding that destruction of both carotenoids is stimulated markedly by the addition of equimolar amounts of *o*-phenanthroline and zinc acetate. The use of crocin as a model carotenoid substrate is further justified by the finding that a purified enzyme fraction which oxidizes crocin will also oxidize β-carotene solubilized with Triton-X-100, and in this case also the destruction is stimulated by the addition of zinc acetate and *o*-phenanthroline.

The experiments using intact chloroplasts show that the destruction of carotenoids is aerobic and is catalyzed by an enzyme which is probably metal-containing. As the destruction of crocin by intact chloroplasts is inhibited much more strongly by potassium cyanide than by sodium diethyldithiocarbamate, it is probable that an iron-containing enzyme is involved.

The stimulation of carotenoid destruction by *o*-phenanthroline and zinc acetate when added together, must be contrasted with the inhibitory effect of *o*-phenanthroline alone and the lack of effect of zinc acetate. A possible explanation is that *o*-phenanthroline removes the metal from the enzyme and that the enzyme then forms a complex with zinc ions; this enzyme–zinc complex being more an effective oxidant of carotenoids than the original enzyme. This explanation is also in accord with the finding that activity is reduced by lowering the ratio of concentration of zinc acetate to *o*-phenanthroline. Although various antioxidants inhibit carotenoid destruction, the mechanism of the inhibition is not clear.

The experiments with catalase and peroxidase indicate that the destruction of carotenoids by chloroplasts is not a coupled oxidation involving hydrogen peroxide. Neither is a coupled oxidation by lipoxidase involved.

Fractionation of a deoxycholate extract of chloroplasts yielded one enzyme fraction which showed a marked stimulation of crocin destruction by *o*-phenanthroline and zinc acetate. It is almost certainly this fraction which is responsible for the observed reactions of intact chloroplasts. There was, however, another fraction which enzymically destroyed crocin but which was inhibited by *o*-phenanthroline and zinc acetate and which was rather unstable.

It is probable that the ratios of these two enzymes may vary considerably during development and ageing of leaves since we have obtained chloroplasts from young leaves which when assayed soon after preparation showed a very high activity towards crocin and were slightly inhibited by *o*-phenanthroline–zinc acetate. After standing for about 2–3 h at + 1°, the activity dropped but the residual activity was then stimulated by *o*-phenanthroline–zinc acetate. It seems that in these chloroplasts there was more of the second enzyme (B_1, Table VII) but that due to its innate instability it disappeared on standing.

The enzymic nature of carotenoid destruction in chloroplasts is clearly established by this study. It is not yet possible, however, to postulate any precise function for these enzymes in the metabolism of the chloroplasts.

ACKNOWLEDGEMENTS

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THE FORM OF CRYSTALS OF MAHONEY POLIOVIRUS GROWN IN PHOSPHATE-SALINE

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SUMMARY

X-ray diffraction studies of wet crystals of Mahoney poliovirus have enabled the crystal morphology to be related to the crystallographic unit cell, which is strictly orthorhombic but approximately body-centred cubic.

The most strongly developed faces are of the form $\{110\}$ which are the most densely packed planes in the virus crystal. These are often bounded by planes of the forms $\{1\bar{1}0\}$ and $\{112\}$ to form short hexagonal prisms. Faces of the forms $\{101\}$ and $\{011\}$ have also been observed, and these tend to give the crystal a truncated-octahedral appearance.

INTRODUCTION

The results of some investigations into the structure of crystals of Type 1 (Mahoney strain) poliovirus have been reported by STEERE AND SCHAFFER[1]. From measurements of the interfacial angles of crystals embedded in clear cement they concluded that the crystals were of cubic form (or nearly so), being octahedral with varying developments of cube faces. This crystal form was correlated with the face-centred cubic arrangement of virus particles observed in electron micrographs of replicas of frozen crystals. Some of these crystals were kindly sent to this laboratory by Dr. SCHAFFER for X-ray crystallographic studies. The crystals had the same general appearance as those described by STEERE AND SCHAFFER although no octahedral crystals were observed. They were too small for optical crystallographic measurements and the shapes of individual crystals varied sufficiently to make the definite determination of the crystal orientation only possible from X-ray diffraction photographs. The crystals are certainly not strictly cubic in symmetry since the larger ones showed a weak birefringence in the polarizing microscope.

The results of the X-ray structure analysis of the wet crystals have already been reported by us[2]. The unit cell is orthorhombic with sides *a*, 353 Å; *b*, 378 Å; and *c*, 320 Å, and the space group is of the type $P2_12_12$ with two virus particles per lattice point, resulting in an approximate body-centred cubic arrangement. As stated in our paper, we attribute the difference between the crystal structure suggested by STEERE AND SCHAFFER and that found by us to the effects of freezing or drying.

Crystal morphology

In the course of the X-ray investigation, we have been able to relate the morphology of the wet crystals to the unit cell. The most common crystal shape was a short, almost regular, hexagonal prism. The crystals tended to rest on one of the hexagonal faces against the walls of the capillary tubes in which they were mounted for the X-ray studies, and clear optical reflections could be obtained from these faces which are of the form $\{110\}$. The "prism" faces must be of the form $\{1\bar{1}0\}$ and $\{112\}$ which are very nearly perpendicular to the (110) and $(\bar{1}\bar{1}0)$ faces as indicated in Fig. 1a. The figure also shows the developments of faces of the forms $\{011\}$ and $\{101\}$ which are also often observed. Photographs of crystals of very similar appearance have been published by STEERE AND SCHAFFER[1], although it is not clear, in the case of the crystal shown in their Fig. 2, whether it was tilted to show the (112) faces or whether the end faces were of the form $\{101\}$ and $\{011\}$.

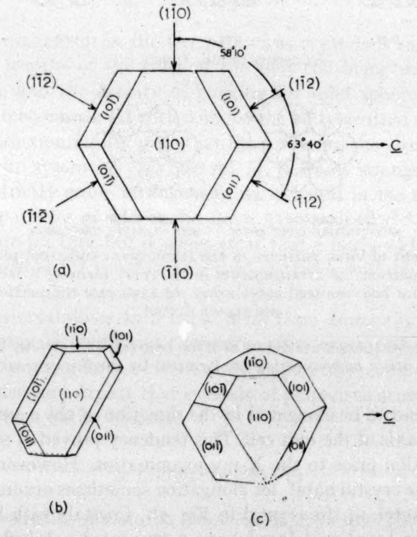

Fig. 1. Sketches of poliovirus crystals, grown from phosphate-saline, drawn in approximately the same orientation. The most common crystal shape is shown in (a). It has large developments of the (110) and $(1\bar{1}0)$ faces and lesser developments of the (112), (101) and (011) faces. The indices of the faces were determined from X-ray diffraction photographs. The crystals are often elongated in the *c* direction, but this is not always found, as shown in (b). When the faces shown are developed to about the same extent, the crystals have a partial-truncated octahedral appearance (c).

It is to be expected that the most strongly developed faces are of the form $\{110\}$, since they correspond to the most densely packed planes of the crystal lattice. In these planes the virus particles are arranged in an almost regular hexagonal array (see Fig. 2), the angle between the nearest neighbours in the directions of the edge and body-diagonal of the unit cell is 58° (compared with 60° for the regular hexagonal array), and the density of packing is 0.97 of that of a close-packed hexagonal array having an interparticle distance[2] of 304 Å. (We are here assuming that the virus particles are in contact in the crystal so that the hydrated particle diameter is equal to the shortest interparticle distance, 304 Å.) After the planes of the form $\{110\}$, the next most densely packed are the $\{011\}$ and $\{101\}$ planes. In these planes (Fig. 2), the angles between the nearest neighbours in the directions of the edge and body-diagonal of the unit cell are approximately $54^\circ 30'$ and $51^\circ 30'$ respectively. The densities of packing are respectively 0.92 and 0.89 of that of the close-packed hexagonal array, the former value being very close to that of the (110) plane of a body-centred cubic arrangement.

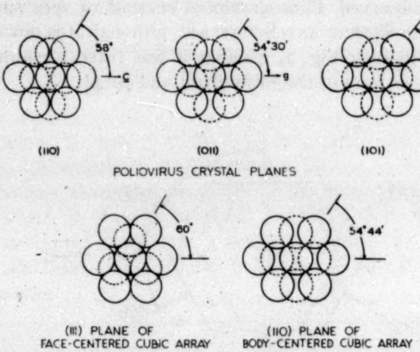
58°
c
54°30'
a
51°30'
b
(110)
(011)
(101)
POLIOVIRUS CRYSTAL PLANES
60°
54°44'
(111) PLANE OF FACE-CENTERED CUBIC ARRAY
(110) PLANE OF BODY-CENTERED CUBIC ARRAY

Fig. 2. The arrangement of virus particles in the (110), (011) and (101) planes of wet poliovirus crystals, and for comparison the arrangements in the (111) plane of a face-centred cubic array and the (110) plane of a body-centred cubic array. In each case the particles in the layer below are shown dotted.

Note. The poliovirus crystal planes are drawn as if the unit cell were strictly body-centred although small departures from strict body-centring are required by the X-ray results[2].

The crystals tend to be elongated in the direction of the *c* axis, possibly because this is the shortest axis of the unit cell. This tendency proved of some help in finding the crystal orientation prior to the X-ray examination. However, this is not a consistent feature of the crystal habit, for elongation sometimes occurs in other directions as shown by the sketch of the crystal in Fig. 1b. Crystals with large developments of faces of the form $\{101\}$ and $\{011\}$ have a truncated octahedral-like appearance (Fig. 1c) which may explain the description of the crystal shape given by STEERE AND SCHAFFER[1]. The values reported by them for the interfacial angles of crystals of this appearance embedded in clear cement are consistent with the truncated-octahedral habit of strictly cubic crystals, but this might well have been produced by the embedding method. In any case, the calculated interfacial angles for the "octahedral" and "cube" faces of the crystal form in Fig. 1c are almost all within 7° of those for a strictly truncated octahedral habit.

Crystal lattice

It is of interest to describe the crystal lattice. The very nearly close-packed (110) planes of the wet poliovirus crystals are not successively close-packed together, but displaced in a direction very nearly perpendicular to the *c*-axis so that each virus particle lies nearly over the point half-way between a pair of particles in the layer below (Fig. 2). The electron micrographs obtained by STEERE AND SCHAFFER show that the virus particles in frozen crystals are close-packed in three dimensions. It thus seems likely that what happens on freezing is a slight rearrangement within the (110) planes to the regular hexagonal array, together with a shear of these planes of about 4°, to the three-dimensional close-packed arrangement. A similar rearrangement has been found in the case of crystals of turnip yellow mosaic virus. In wet crystals of this virus the virus particles are arranged on a body-centred cubic lattice[3], whereas electron micrographs of dried crystals show a face-centred cubic arrangement[4]; and such an arrangement has been observed in all electron micrographs of crystals of the small spherical viruses so far obtained[5].

DISCUSSION

The particle arrangement in the wet poliovirus crystals is rather close to body-centred cubic, the lengths of the sides of the unit cell being within 10 % of their mean value, 350 Å, and the density of packing for solid spheres in the poliovirus arrangement being 0.69 compared with 0.68 for the body-centred cubic arrangement. Since the X-ray diffraction results show that the surface of the virus particle possesses the cubic point group symmetry 532 (see ref. 2), it seems strange that the particles do not pack in a strictly cubic arrangement as is found in the cases of crystals of other viruses, the particles of which also have 532 symmetry[3,6]. We are unable to offer any explanation for this, but it seems clear that a fairly wide variety of crystal forms might be possible for roughly spherical particles without strong directional interactions, the particular lattice adopted depending on the solvent. Thus turnip yellow mosaic virus crystallises in a cubic form from ammonium sulphate[3] and in a non-cubic form from alcohol[7], and Coxsackie virus crystals[8,9] grow in an orthorhombic form from ammonium acetate and an apparently cubic form from normal saline. We would therefore expect that crystals of poliovirus grown from some other solvent might well turn out to be cubic.

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EFFECT OF MITOMYCIN C ON THE SYNTHESIS OF BACTERIAL AND VIRAL DEOXYRIBONUCLEIC ACID

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SUMMARY

1. Treatment of *Escherichia coli* B with 10 μg/ml of mitomycin C resulted in a complete block of DNA formation, although it caused no inhibition of either RNA or protein synthesis. Acid-soluble deoxyribosidic compounds accumulated in the bacterial culture thus treated with mitomycin C.

2. DNA synthesis by bacteria infected with T2r phage was not inhibited by mitomycin C at a concentration sufficient to inhibit DNA synthesis in uninfected organisms. Mitomycin C-treated bacteria resumed DNA synthesis also when infected with either T3 or T5 phage.

3. Although mitomycin C did not affect the infectivity of free phage, the reproduction of phage was progressively impaired with increasing concentrations of the antibiotic. Phage-infected bacteria in contact with high levels of mitomycin C (100 μg/ml) synthesized DNA, released it into the culture medium by lysis but produced no infective particles.

4. It was suggested that virus infection opened a new pathway of DNA synthesis which was originally absent in the uninfected cells and through which the viral DNA was produced in the presence of mitomycin C.

INTRODUCTION

It has recently been shown that mitomycin C (MC), a new antibiotic from *Streptomyces caespitosus*, exhibited selective inhibitory action on DNA formation without affecting the formation of either RNA or protein in *Escherichia coli*[1], as well as a marked antitumor effect[2]. Additional evidences were obtained that this antibiotic has the ability to exert a mutagenic action[3] and to induce the development of active lambda phage from the prophage stage in the lysogenic strain of *E. coli* K-12[4]. These properties are similar to ultraviolet effects, and it therefore appears that the nuclear apparatus of the cells is particularly susceptible to the action of MC. Hence analysis of the mode of MC action may be of great aid in understanding the mechanisms of DNA synthesis and of the effect of radiation on cell reproduction.

Abbreviations used: MC, mitomycin C; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

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A number of investigations on DNA synthesis in phage-infected bacteria have been performed in an attempt to understand the nature of viral infection and to extend our knowledge of the mechanism of DNA replication in the cells. Recent enzymic studies revealed that phage infection induced new enzymes which catalysed the formation of the compounds, such as 5-hydroxymethylcytidylic acid, peculiar to viral DNA[5-7]. A curious phenomenon has been observed that the impaired synthesis of DNA in bacteria treated with MC can be promptly restored by infection with T2r phage[8]. The present paper concerns the detailed studies on the different behaviour of MC on the DNA synthesis in phage-infected and uninfected cells.

MATERIALS AND METHODS

Chemicals

The crystalline MC was supplied by the Kyowa Fermentation Industry Co., Ltd., Tokyo, through the courtesy of Dr. S. SHIBA of Osaka University, to whom our thanks are due.

Bacterial and phage strains

Most of the experiments were done with the following two strains of *E. coli* B: Strain B(H) originally derived from the laboratory of Dr. A. D. HERSHEY was obtained from Dr. I. WATANABE of Kyoto University. A MC-sensitive strain B_{ms} was found in the collection of the Research Institute for Microbial Diseases, Osaka University. The bacteriophage strains studied were T2, T3 and T5; these were also furnished by Dr. I. WATANABE. The T2 phage was the rapid-lysing mutant *r*.

Growth of bacteria

Bacteria were grown in a glucose-salts synthetic medium containing (per litre) 2.5 g of $(NH_4)_2HPO_4$, 1.5 g of KH_2PO_4, 5 g of NaCl, 3 g of sodium glutamate, 3 g of glucose, and 0.1 g of $MgSO_4 \cdot 7H_2O$ at pH 7.2 In experiments with phage T5, the medium was supplemented with $CaCl_2$ at a concentration of $1 \cdot 10^{-4}$ *M*[9]. The cultures were incubated at 37° overnight; then, after diluting the entire culture into fresh medium, the cells were reincubated with vigorous aeration. Growth was followed by measurement of the optical density at 660 mμ. When the bacteria, growing logarithmically, had attained an optical density of 0.3 corresponding to $5 \cdot 10^8$ cells/ml, they were harvested by centrifugation and resuspended in fresh medium.

The basic procedures employed in the preparation of phage stock and in the assay of infectivity of phage were those described by ADAMS[10]. Viable cells were counted by plating on nutrient agar.

Analytical methods

Determinations of nucleic acid and protein were carried out as follows: 10 ml aliquots of the bacterial suspension (at a titre of $5 \cdot 10^8$ cells/ml) were chilled and rapidly acidified by addition of 10 *N* perchloric acid to give a final concentration of 0.5 *N*. After standing for 30 min in the cold, the insoluble residue was collected by centrifugation and then heated with 0.5 *N* perchloric acid at 90° for 15 min in order to extract nucleic acids. DNA and RNA in the extracts was determined by the method of BURTON[11] and of MEJBAUM[12], respectively. The residue remaining after extraction of nucleic acids was dissolved in a small volume of 1 *N* NaOH, and protein was determined according to the method of LOWRY *et al.*[13].

Bacterial cultures were extracted in the cold with two successive portions of 0.5 *N* perchloric acid for the assay of the acid-soluble fraction. The combined extracts were neutralized, divided into two portions, and each was incubated at 37° overnight in the presence or absence of snake venom dissolved in maleic acid–KOH buffer (pH 6.5) containing 10^{-2} *M* $MgSO_4$. Deoxyribosidic compounds were determined in the venom-treated and untreated extracts by means of the growth of *Lactobacillus acidophilus* R26, as described by OKAZAKI *et al.*[14].

RESULTS

Inhibition of DNA synthesis by mitomycin C

The effects of MC on the synthesis of nucleic acids and protein in *E. coli* strain B are shown in Fig. 1. The net synthesis of DNA was completely inhibited in the presence of MC at a concentration of 10 μg/ml, while RNA and protein formation were unaffected or only slightly reduced as compared with that of a culture not exposed to the antibiotic. A marked reduction of the viable count was observed after exposure to MC. In addition, bacteria exposed to MC for a short time could not synthesize DNA after removal of the antibiotic from the medium (Fig. 2) and there was a slight decline in the DNA contents of exposed bacteria. These observations are in full agreement with the previous work of SHIBA *et al.*[1]. The results prompted us to search for effects on the deoxynucleotide pool. In experiments conducted as described

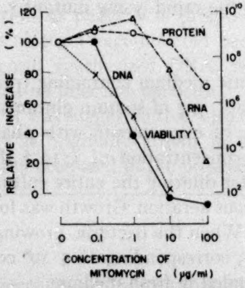

Fig. 1. Effects of MC on nucleic acid and protein synthesis by *E. coli* B. Tubes containing 10 ml of cell suspension ($5 \cdot 10^8$ cells/ml) in the growth medium to which was added various levels of the antibiotic as shown, were incubated with shaking at 37° for 30 min. The relative rate of increase of the constituents during the test period is indicated in the figure. The increment of the control, incubated in the absence of MC, is represented by 100.

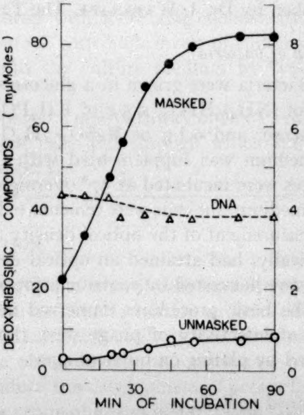

Fig. 2. The DNA and acid-soluble deoxyribosidic compound content of *E. coli* B culture subjected to MC treatment. Bacteria were exposed to 10 μg/ml of MC in glucose–salts medium for 5 min at 37°, centrifuged and washed twice with saline. The cells were then reincubated in fresh medium in the absence of antibiotic and 10 ml of aliquots of the culture ($5 \cdot 10^8$ cells/ml) were taken for assays at the time points indicated. "Unmasked" or "masked" indicates the deoxyribosidic compounds responsible for the growth of *Lactobacillus acidophilus* R26 before and after digestion with a snake venom preparation.

in the experimental section, treatment of the cells with MC rapidly raised the level of the deoxyribosidic compound content of the acid-soluble fraction of the culture. This increase in deoxyribosidic compounds reached a maximum after about 60 min of incubation (Fig. 2). This phenomenon bears a resemblance to the behaviour of *E. coli* subjected to ultraviolet irradiation[15] or mustard treatment[16]. Furthermore, the major portion of the accumulated compounds was observed to be masked deoxyribonucleotide that required a preliminary digestion with snake venom to support the growth of the test organism similar to that found previously in the acid-soluble extracts of sea urchin eggs[17] and *Lactobacillus*[14].

DNA synthesis in phage-infected bacteria

The synthesis of DNA by T2r phage-infected bacteria in the presence of MC is illustrated in Table I. Bacteria infected with T2r phage could synthesize DNA at the normal rate even at a level of 10 μg/ml of antibiotic. This MC concentration is sufficient to abolish the increase of DNA in uninfected cells. A somewhat lower rate of synthesis of DNA was obtained at higher concentrations of MC, *i.e.*, 100 μg/ml.

TABLE I

DNA SYNTHESIS BY T2r PHAGE-INFECTED AND UNINFECTED *E. coli* B CELLS IN THE PRESENCE OF MITOMYCIN C

Cells harvested during the logarithmic phase were resuspended in fresh medium at a concentration of $5 \cdot 10^8$ cells/ml and divided into two portions. One portion of the bacterial suspension was infected with a five-fold multiplicity of T2r phage and the other portion was kept uninfected as a control. Tubes containing 10 ml of a suspension of infected or uninfected cells were incubated with various levels of MC as described in the table at 37° for 30 min, and analysed for DNA.

Time of incubation (min)	*Concentration of MC (μg/ml)*	*Uninfected cells*		*T2r-infected cells*	
		Relative amount of DNA	*Increment of DNA (% of control)*	*Relative amount of DNA*	*Increment of DNA (% of control)*
0	0	100	—	100	—
30	0	149	100	288	100
30	0.1	150	102	286	99
30	1	113	28	284	98
30	10	99	< 0	278	95
30	100	97	< 0	165	35

Of great interest is the observation that synthesis of DNA was resumed upon phage infection of bacteria pretreated with MC (Fig. 3). When cells which had lost their ability to form DNA as a result of MC treatment at a level of 10 μg/ml for 30 min, were infected with T2r phage, they abruptly began to form DNA even though the antibiotic was still present in the culture medium. The rate of DNA production in the treated infected bacteria was exactly the same as that in the untreated organisms. In order to examine the possibility that the inhibitory action of MC might be reduced during the period of pretreatment, medium containing 10 μg/ml of MC was incubated at 37° for 60 min with *E. coli* strain B added at a titre of $5 \cdot 10^8$ cells/ml, centrifuged and then the supernatant culture medium was reincubated with fresh bacteria. MC thus pretreated was as effective in suppressing DNA synthesis as was untreated material, suggesting that the antibiotic was not inactivated during incubation with the bacterial culture, and that the process of DNA synthesis in phage-infected cells is really resistant to MC inhibition.

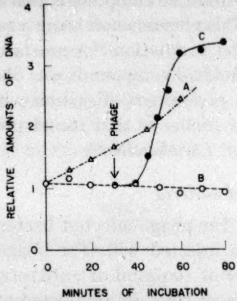

Fig. 3. Resumption of DNA synthesis by MC-treated *E. coli* B on phage infection. A growing culture of *E. coli* B was divided into three portions and incubated at 37° under the conditions described as follows: A, in the absence of MC; B, in the presence of 10 μg/ml of MC; C, in the presence of 10 μg/ml of MC for 30 min, after which T2r phage was added at a multiplicity of 5. Aliquots of the suspension were taken at intervals as indicated and analysed for DNA.

Since the DNA of phage T2 differs from that of the host bacterium in containing 5-hydroxymethylcytosine in place of cytosine[18], it seemed possible that the resistance of infected cells to MC inhibition might be due to the absence of cytosine in phage DNA. Therefore we determined whether infection with a T-odd numbered phage containing cytosine rather than 5-hydroxymethylcytosine in its DNA molecule would cause the resumption of MC-inhibited DNA synthesis. The results with T3 or T5 phage are summarized in Table II and indicate that DNA synthesis in infected cells can proceed without any inhibition in media containing 10 μg/ml of MC, as is observed in the case of T2r phage infection. Therefore the presence of 5-hydroxy-

TABLE II

EFFECT OF MITOMYCIN C ON DNA SYNTHESIS BY *E. coli* B INFECTED WITH T3 OR T5 PHAGE

Experimental procedures as described in Table I.

Phage	*Time of incubation (min)*	*Concentration of MC (μg/ml)*	*Uninfected cells*		*Infected cells**	
			Relative amount of DNA	*Increment of DNA (% of control)*	*Relative amount of DNA*	*Increment of DNA (% of control)*
T3	0	0	100	—	100	—
	20	0	130	100	113	100
	20	1	121	70	113	100
	20	10	99	< 0	112	92
	20	100	96	< 0	109	69
T5	0	0	100	—	100	—
	60	0	234	100	413	100
	60	1	174	55	415	101
	60	10	110	7	383	91
	60	100	89	< 0	155	18

* Each phage was added at a multiplicity of 5.

methylcytosine in phage DNA does not seem to be responsible for the high resistance to MC of DNA synthesis of phage-infected cells. This resistance to MC does not seem to be the result of a high rate of DNA formation in the infected cells, since there was little net synthesis of DNA in T3 phage-infected cells.

Effect of mitomycin C on phage reproduction

As a result of the preceding experiments it was of interest to study the effect of MC on phage reproduction. The exposure of free phage particles to MC did not cause any inactivation, judging by the infectivity of a phage suspension incubated with MC (Table III, Expt. I). However, the reproduction of phage T2r was progressively impaired with increasing concentrations of MC. Although the multiplicity of infection for phage reproduction in Expt. II was extremely low, similar results were obtained in Expt. III, in which a multiplicity of 5 was used which was the multiplicity used in the chemical determinations. It must be emphasized that there is an increase in phage titre in the presence of MC at a concentration sufficient to inhibit DNA synthesis completely in uninfected bacteria (*e.g.* 10 μg/ml), indicating that a portion of the phage produced in T2r-infected bacteria under these conditions is in biologically active form.

At higher concentrations of MC (*e.g.* 100 μg/ml), the culture of infected organisms became clear by lysis of the cells, but did not give rise to infective phage. Furthermore, as shown in Table IV, the bacteria infected in the presence of MC release DNA to the low speed supernatant fraction of the culture at a rate comparable to that of the control infected in the absence of MC. In the uninfected bacteria almost all of the DNA is found in the precipitate, both in the presence or in the absence of MC.

TABLE III

EFFECT OF MITOMYCIN C ON THE REPRODUCTION OF T2r PHAGE

Expt. No.	*Time of incubation (min)*	*Concentration of MC (μg/ml)*	*Phage titre (per ml)*	*Relative increase of phage titre (% of control)*
I[*]	0	0	$5.8 \cdot 10^4$	—
	60	0	$5.6 \cdot 10^4$	—
	60	10	$5.9 \cdot 10^4$	—
	60	100	$5.6 \cdot 10^4$	—
II[**]	0	0	$1.0 \cdot 10^5$	—
	30	0	$1.1 \cdot 10^7$	100
	30	0.1	$1.2 \cdot 10^7$	109
	30	1	$6.9 \cdot 10^6$	62
	30	10	$2.4 \cdot 10^6$	21
	30	100	$< 2.0 \cdot 10^3$	< 0
III[**]	0	0	$2.4 \cdot 10^9$	—
	30	0	$1.9 \cdot 10^{10}$	100
	30	10	$5.2 \cdot 10^9$	17
	30	100	$1.9 \cdot 10^9$	< 0

[*] Phage was suspended in the glucose–salts medium containing MC at a level shown in the table and incubated at 37° without addition of bacterial suspension.

[**] Phage suspension was shaken at 37° with $5 \cdot 10^8$ cells/ml of *E. coli* B suspended in the medium containing MC. Actual multiplicity of infection was 1 for Expt. II (average multiplicity of 0.0002), and 5 for Expt. III, respectively.

TABLE IV

LYSIS OF T2r PHAGE-INFECTED *E. coli* B IN THE PRESENCE OF MITOMYCIN C

One portion of the cell suspension ($5 \cdot 10^8$ cells/ml) infected with a ten-fold multiplicity of T2r phage and another not infected were incubated at 37° for 60 min with shaking. The cultures were then chilled and centrifuged for 10 min at 6,000 rev./min in the cold.

Bacteria	*Concentration of MC (µg/ml)*	*Amount of DNA-P (µg)*		*Sup./Total (%)*
		Supernatant	*Total*	
Uninfected	0	0.3	20.4	1
	10	0.3	9.0	3
	100	0.2	9.0	2
T2r-infected	0	26.4	46.1	57
	10	25.6	46.0	56
	100	12.9	28.8	45

TABLE V

EFFECT OF MITOMYCIN C ON THE MULTIPLICATION OF PHAGE

Concentration of MC (µg/ml)	*Increment in phage titre* (% of control)*		
	T2r	*T3*	*T5*
0	100	100	100
0.1	109	105	109
1	62	47	50
10	21	29	20
100	< 0	< 0	2

* T2r, T3 and T5 phage were incubated with the bacterial suspension for 20, 30 and 60 min, respectively. Experimental conditions as described in Table III, Expt. II.

Supernatant fraction of lysate obtained from culture incubated in the presence of a high level of MC contains about half of the DNA found in the untreated control, but the infectivity of such a lysate is only one-fiftieth to one-hundredth of the latter, suggesting that the DNA produced in the presence of the antibiotic is practically all the non-infectious form. Similar results were obtained with T3 or T5 phage, and the reproduction of these phages was similarly impaired by MC (Table V).

DNA synthesis in mitomycin C-sensitive bacteria

Comparison of the resistance of various *E. coli* B strains to the inhibitory action of MC revealed that the particular strain used by SHIBA *et al.*[1] was the most sensitive to the antibiotic. DNA formation in this strain was depressed by about one–hundredth of the amount of MC required to inhibit DNA formation in the other strains; RNA and protein synthesis was also strongly reduced in the presence of 10 µg/ml of MC (Fig. 4). This amount of MC causes the selective inhibition of DNA production in the strain B. Thus this strain was subsequently referred to as strain B_{ms}. It was noticed that strain B_{ms} was more sensitive to ultraviolet irradiation and to nitrogen mustard treatment than was the other strain, although the strain B_{ms} was not more sensitive to other antibiotics such as chloramphenicol and streptomycin*.

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* Details on the relationship between resistance to MC and radiation will be published elsewhere.

The comparative effect of MC on DNA synthesis in B and B_{ms} strains is summarized in Fig. 5, which indicates that the MC-sensitive strain B_{ms} acquires the ability to make DNA in the presence of MC on infection with phage T2r as does the other strain. DNA synthesis by infected B_{ms} is influenced less by the addition of MC than is uninfected B but is apparently more sensitive than infected B. At a concentration of 10 μg/ml MC did not effect the formation of DNA in infected B but slightly inhibited DNA formation in infected B_{ms}. It was suspected that MC might inhibit the formation of protein essential for the initiation of DNA synthesis by phage-infected bacteria. Therefore an experiment in which the antibiotic was added at various times after infection was performed since it is known that protein formation must precede DNA formation in infected bacteria. MC added after the maximum rate of DNA synthesis had been attained, rapidly suppressed further production (Fig. 6), indicating that MC inhibits DNA synthesis itself rather than the initiation of this process.

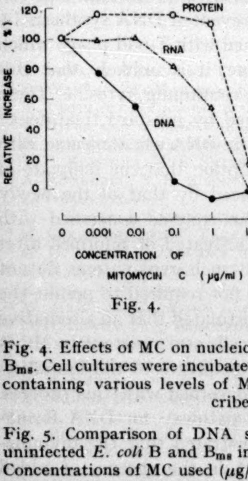

Fig. 4.

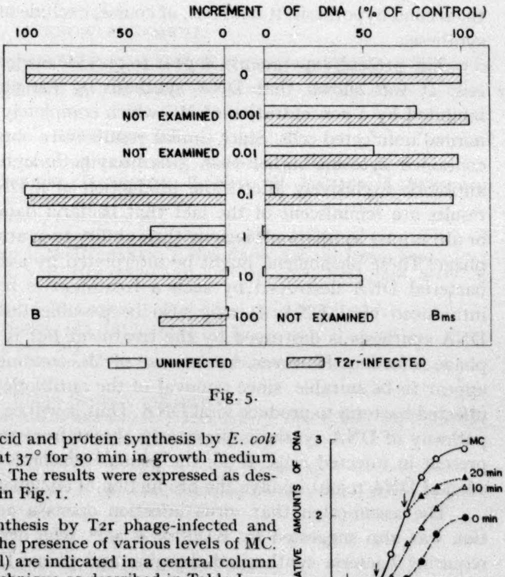

Fig. 5.

Fig. 4. Effects of MC on nucleic acid and protein synthesis by *E. coli* B_{ms}. Cell cultures were incubated at 37° for 30 min in growth medium containing various levels of MC. The results were expressed as described in Fig. 1.

Fig. 5. Comparison of DNA synthesis by T2r phage-infected and uninfected *E. coli* B and B_{ms} in the presence of various levels of MC. Concentrations of MC used (μg/ml) are indicated in a central column in the figure. Experimental technique as described in Table I.

Fig. 6. Effect of the delayed addition of MC on DNA synthesis by T2r phage-infected *E. coli* B_{ms}. The cells were infected by T2r phage with an average multiplicity of 5 at time 0, incubated with shaking and MC was added at time points indicated by arrows to give a final concentration of 10 μg/ml.

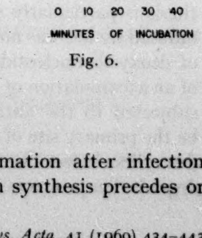

Fig. 6.

DISCUSSION

Protein synthesis is essential to the initiation of DNA formation after infection with T2 phage[19-21]. It has also been shown that this protein synthesis precedes or accompanies formation of a RNA peculiar to phage-infected cells[22, 23], in agreement with the current view that RNA is involved in protein formation. The early synthesized protein(s) does not seem to be the antigenic protein associated with the virus structure, since the latter protein first appears in the infected cell about 10 min after infection[24, 25]. There are several interpretations of the function of protein formation in the initiation of viral DNA synthesis. One possibility is that newly synthesized protein transfers genetic information to progeny from parental DNA[26, 27]. The second hypothesis implies that the protein is a mixture of enzymes which synthesize compounds, such as 5-hydroxymethylcytidylic acid, peculiar to phage DNA. Finally, one may suppose that in infected cells there is an alternative pathway of DNA formation developed in infected cells and that this pathway is absent from the normal cells, or present in insufficient amount. It has recently been shown that some enzymes which catalyse the formation of a phage specific pyrimidine nucleotide are induced in T-even phage-infected cells[5–7]. Although these works seem to favour the second hypothesis, it does not, of course, exclude other roles for the initial protein synthesis.

The present experiments appear to provide evidence to support the last hypothesis. It was shown that DNA synthesis by T2r phage-infected bacteria was not inhibited by a concentration of MC which completely prevented DNA synthesis by normal uninfected cells. Since similar results were obtained with T-odd phage which contained cytosine rather than 5-hydroxymethylcytosine, it is unlikely that this antibiotic exclusively affects the production of a DNA containing cytosine. These results are reminiscent of the fact that bacteria damaged by mustard treatment[28] or ultraviolet irradiation[29] recover their ability to synthesize DNA when infected with phage. These phenomena might be interpreted by assumption that the template of bacterial DNA destroyed by such a treatment is replaced by that of the newly introduced viral DNA. It may also be possible that an enzyme concerned with DNA synthesis is destroyed by the treatment but is reactivated or reformed after phage infection. However, in the case of MC treatment, such explanations do not appear to be suitable, since removal of the antibiotic is not required to permit the infected bacteria to produce viral DNA. Thus it will be postulated that an alternative pathway of DNA synthesis previously absent from the cells and resistant to MC is present in infected cells. If so, the protein synthesis prerequisite to the formation of viral DNA might involve the production of enzymes concerned with this process.

The assumption that virus infection opens a new pathway for DNA formation was also suggested by BARNER *et al.*[30], who demonstrated that the thymine-requiring bacteria synthesized thymine and phage DNA in the absence of added thymine in the medium on infection with the phage. Similar observations have recently been reported with T-odd phage[31]. The problem is what step of DNA synthesis is particularly susceptible to the action of MC. The block in DNA synthesis induced by MC was not overcome by the addition of thymine, thymidine or a mixture of deoxyribonucleotides to the medium[32]. These results, together with our finding of an accumulation of deoxyribonucleotides in the acid-soluble fraction of the culture subjected to the antibiotic, seem to suggest that the DNA polymerizing step may be the primary site of attack by MC. However, it is not possible to state whether the deoxyribonucleotides accumulated represent precursors of DNA or acid-soluble degradation products. Furthermore, preliminary experiments in our laboratory have indicated that the incorporation of ^{32}P-labelled deoxyribonucleotides into DNA by a cell-free extract obtained from uninfected cells by the method of Kornberg was not suppressed in the presence of MC at the low level used for the experiments with intact cells[32]. It is known that the DNA polymerizing enzyme catalyses the formation of viral DNA as well as bacterial DNA[5,6]. Therefore the site of the action of MC on DNA formation presents a confusing picture, suggesting that the process of DNA formation in intact cells is very complex.

A further interesting fact is that the phage produced in the presence of a high level of MC is apparently non-infectious. In the subsequent experiments it was demonstrated that the non-infectious phages produced by the action of MC had an ability to adsorb to sensitive bacteria and to inject their DNA into the cells which they attached[33]. Therefore the low infectivity of these phages is possibly attributed to the biological inactivity of their DNA, suggesting that the viral DNA produced in the presence of a high level of MC is abnormal.

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STUDIES ON CYTOPLASMIC RIBONUCLEIC ACID FROM RAT LIVER

I. FRACTIONATION AND FUNCTION OF SOLUBLE RIBONUCLEIC ACID

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SUMMARY

1. The soluble polynucleotides isolated by phenol extraction from rat-liver cytoplasm were fractionated on the resin Ecteola.

2. Four fractions were obtained, one of which (s_3-RNA) could be transferred to microsomal RNA in the presence of soluble enzymes, ATP, GTP and a nucleoside triphosphate regenerating system. The efficiency of this transfer was about 17 % based on the isotope content of the added polynucleotides.

3. On incubation of pH 5 enzymes with DL-[^{14}C]leucine or a mixture of radioactive amino acids in the presence of ATP, s_3-RNA became mainly labelled. Minor amounts of radioactivity, not exceeding 10 % of that recovered in s_3-RNA, were also detected in other fractions.

4. The fractions were further characterized by ultracentrifugal analysis, dialysis and treatment with EDTA.

5. The possible significance of these results for the mechanism of protein biosynthesis in rat-liver microsomes is discussed.

INTRODUCTION

The suggestion[1] that soluble poly- and oligonucleotides play an essential role in the biosynthesis of proteins in liver microsomes has gained experimental support as has been shown by several authors[2-6].

The preliminary activation of the amino acids by ATP and specific enzymes is followed by an attachment of these acids to the terminal nucleotide of soluble RNA through ester linkages[7-9]. Incubation of this s-RNA-amino acid complex with liver microsomes in the presence of GTP and ATP results in the incorporation of amino acid into microsomal ribonucleoprotein[2]. These conditions also favour a metabolic transfer of soluble polynucleotides to microsomal RNA[10-15]. Although a GTP dependence has been demonstrated for the latter two processes[2, 10-14] it is not known

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Abbreviations used: s-RNA, soluble ribonucleic acid; m-RNA, microsomal ribonucleic acid; AMP, adenosine monophosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; PCA, perchloric acid; NAA, nicotinamide; EDTA, ethylenediaminetetraacetate; RNP, ribonucleoprotein.

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to which extent the two transfer reactions are coupled. Fractionation and purification of the enzymes as well as that of the soluble polynucleotides involved are necessary. In the present paper s-RNA has been resolved in four fractions by chromatography on Ecteola and the metabolic capacities of the fractions so obtained have been compared.

MATERIALS AND METHODS

Preparation of pH 5 enzymes, microsomes, and nucleoprotein particles

Rats of the R strain, fed *ad libitum*, were killed by decapitation following light anaesthesia. The livers were transferred immediately afterwards to a beaker containing a small volume of homogenization medium (medium A) which was placed in crushed ice. The livers were minced with scissors and homogenized in 2.5 volume of a buffer containing 0.02 M potassium phosphate buffer, pH 7.8, 0.025 M KCl, 0.05 M Tris, 0.35 M sucrose, 0.01 M NAA, 0.01 M $MgCl_2$ (medium A[16,17]). Homogenization was carried out in a glass homogenizer at a speed of the rotating pestle of 500 rev./min, which was controlled electrically. The homogenate was then centrifuged at 15,000 × g for 10 min in a cooled Phywe centrifuge. The upper half of the supernatant was used as such in some of the incubation experiments (15,000 × g supernatant). For the isolation of microsomes and pH 5 enzymes the 15,000 × g supernatant was centrifuged at 105,000 × g for 1.5 h in rotor 40 of the Spinco ultracentrifuge, Model L. The upper half of the supernatant was removed and used further (105,000 × g supernatant), the remainder discarded. The microsomal pellet was covered with a small volume of medium A, which was then drained off. The pellet obtained in this way served only as a starting material for the isolation of nucleoprotein particles, but was never used in the incubation experiments. The nucleoprotein particles were obtained by dispersing the microsomal pellet in 0.5 % deoxycholate in 0.05 M glycylglycine buffer of pH 8 (according to LITTLEFIELD *et al.*[18]), adding distilled water as described. The resulting suspension was then centrifuged for 2 h at 105,000 × g, yielding a transparant pellet covered with a dense zone of brown material and a rather loose sediment. The relative amounts of dense and loosely packed material seemed to vary somewhat with the age of the animals. If the radioactivity had to be determined in the microsomal RNA the two upper parts of the sediment were suspended in 10 % NaCl leaving the glycogen pellet behind.

In most of the experiments the 105,000 × g supernatant was used as such; pH 5 enzymes were obtained by lowering the pH to 5.2 by means of 1 % acetic acid at 2–4° (see ref. 13). Following centrifugation at 3000 × g for 10 min at 2–4°, the supernatant was removed. The sediment was washed once with cold medium A and then redissolved in an appropriate volume of cold medium A. In general, material insoluble at pH 7.4 was removed by centrifugation and discarded.

For those experiments in which microsomes were incubated, a different isolation technique was followed. The livers were homogenized in medium B (composed of 1 volume of medium A and 1 volume of redistilled water). The 15,000 × g supernatant which was obtained in a similar way as described above was then layered over 20 ml of cold medium A in 30 ml centrifuge tubes. Centrifugation was then carried out in the rotor No. 30 at 78,000 × g average for 1 h. The microsomal sediment was carefully resuspended in medium A and centrifuged for some minutes at 500 × g.

The milky supernatant was separated from the sediment and used as such. The technique follows essentially the prescriptions of HULTIN *et al.*[10, 11].

Phenol extraction of RNA

The phenol extraction[19,20] was performed twice at 2–4° and the separation of the two layers was achieved by centrifugation in a cooled centrifuge. The clear solution was extracted 3 times with ether and RNA was precipitated by adding 3 volumes of ethanol and 0.1 volume of 20 % potassium acetate. Higher amounts of RNA were consistently obtained at 2–4° than at 20°.

Isolation of microsomal RNA for radioactivity assay

Ribonucleoprotein particles, isolated by deoxycholate treatment of the liver microsomes[18], were suspended in 10 % NaCl, taking care that glycogen remained behind as much as possible. The suspension was heated in a boiling water bath for 30 min and the extract was cleared from protein by centrifugation and filtration through cotton wool. The protein residue was re-extracted with a smaller volume of 10 % NaCl, the joined extracts were supplemented with 3 volumes of ethanol and left overnight at —15°. The RNA precipitate was isolated by centrifugation and redissolved in a small volume of water. One aliquot of this solution was plated for radioactivity assay, another was hydrolyzed with PCA and assayed for RNA by reading O.D. at 260 mμ (see ref. 21).

Starch block electrophoresis

Zone electrophoresis on starch was performed as described elsewhere[22,23]. The starch block (40 × 2 × 4 cm) was kept in a vertical position so as to counterbalance electrosmotic flow by gravitational forces. The temperature (2–4°) was controlled by measuring the electrical resistance of a thermistor, which was inserted into the starch block. Electrophoresis was carried out for 16 h at 4.5 V/cm and 10–12 mA.

Chromatography on Ecteola

The weak anion exchanger Ecteola was prepared from Whatman cellulose powder according to PETERSON AND SOBER[24]. Different batches were prepared but most of the experiments described in this and the subsequent paper were performed with a resin which had a binding capacity of 0.26 meq. acid/g (determined by potentiometric titration) and a nitrogen content of 0.28 % (determined by Kjeldahl analysis, compare[25]). Columns of 4 cm and a diameter of 1 cm, containing about 500 mg dry wt. of exchanger, were washed extensively with 1 *N* NaOH for 18 h or more, followed by 0.01 *M* NaH_2PO_4 buffer of pH 5 until the pH of the effluent was 5. The columns were charged with 1–3 mg RNA in 0.01 *M* phosphate buffer and eluted as described in the text and the legends of Fig. 1. Light absorbance of the effluent at 253 mμ was recorded continuously as described elsewhere[26].

Chemical analyses

Protein content was determined according to LOWRY *et al.*[27]. The RNA was eluted from the starch segments, hydrolyzed with 5 % $HClO_4$ at 100° for 15 min and assayed by reading O.D. at 260 mμ. The RNA content of tissue fractions was estimated by the method of SCOTT *et al.*[28].

Following chromatography, the various fractions were assayed for RNA by reading O.D. without previous hydrolysis. NaCl concentration was assayed by Mohr's titration.

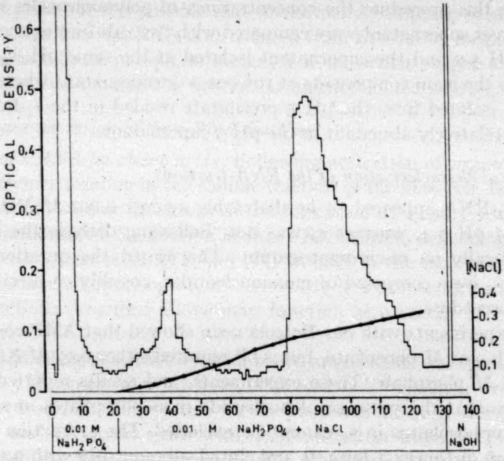

Fig. 1. Fractionation of s-RNA on Ecteola. A gradient elution system was established by applying a stock of 1 *M* NaCl–0.01 *M* phosphate (pH 5) solution and a mixing flask containing 1 l of 0.01 *M* phosphate (pH 5).

RESULTS

Fractionation of soluble RNA on Ecteola

s-RNA was isolated from rat-liver cytoplasm in two ways: (a) by phenol extraction of a 105,000 × *g* liver supernatant or of pH 5 enzymes[19,20]; (b) by zone electrophoresis on starch of pH 5 enzymes as described in an earlier paper[23]. The ribonucleoproteins which precipitate from a 105,000 × *g* liver supernatant on lowering of the pH to 5.2 dissociate into s-RNA and protein. The dissociation is reversed when the pH is restored to 7.4. The so-called pH 5 enzymes, which are obtained by removing the supernatant at pH 5.2 and redissolving the precipitate at pH 7.4, contain s-RNA which is almost completely free and is easily separated from protein by zone electrophoresis on starch.

s-RNA isolated in either way could be fractionated on the weak anion exchanger Ecteola[24,25], yielding essentially identical results (Fig. 1). Four fractions were obtained, designated s_1-, s_2-, s_3-, and s_4-RNA, respectively. The first fraction represented material which was not retained by the resin and ran through the column in 0.01 *M* phosphate buffer of pH 5. s_2- and s_3-RNA were isolated by gradient elution with NaCl in the same phosphate buffer, whereas s_4-RNA was finally removed by eluting with 0.1 or 1.0 *N* NaOH. This elution pattern was strictly reproducible for each column of Ecteola prepared from the same batch, but varied from batch to batch.

A preliminary washing of the column before use with 0.004 *M* EDTA in 0.01 *M* phosphate led to a sharpening of the zones but did not alter the NaCl concentrations at which the various fractions could be eluted. Once these salt concentrations were established the gradient elution was abandoned and small columns were operated manually and washed with NaCl solutions of fixed concentrations.

Following this procedure the concentrations of polynucleotides residing in the 105,000 × *g* liver supernatant were compared with two sub-fractions: the precipitate obtained at pH 5.2 and the supernatant isolated at the same pH. s_2- and s_3-RNA appeared to be the main components of 105,000 × *g* supernatant whereas about 80% of the s-RNA isolated from the pH 5 precipitate resided in the s_3-fraction. s_1- and s_2-RNA were relatively abundant in the pH 5 supernatant.

Physico-chemical characterization of the RNA-fractions

s_1- and s_2-RNA appeared to be dialysable against 0.001 *M* $MgCl_2$ solution in 0.01 *M* Tris of pH 7.4, whereas s_3 was not. Following dialysis the latter fraction behaved identically on re-chromatography. This raised the question as to which extent s_1 and s_2 were composed of mononucleotides, possibly co-precipitated during the isolation procedure.

Control experiments with our Ecteola resin showed that AMP could be isolated by elution with 0.01 *M* phosphate, but ATP required either 0.07 *M* NaCl or 0.004 *M* EDTA in 0.01 *M* phosphate. These experiments, and results reported by BENDICH *et al.*[25] indicate that the presence of nucleoside monophosphates in s_1, and that of nucleoside polyphosphates in s_2 cannot be excluded. The s_1-fraction was therefore put on a column of Dowex-1-formate and eluted subsequently with 0.1 *M* and 0.5 *M* formic acid, 0.1 and 0.2 *M* ammonium formate, and 2 *M* formic acid. The s_1-fraction was almost completely retained under these conditions, 3% being eluted by means of 0.1 *M* formic acid. This result offers evidence that the s_1-fraction contains only a few per cent of mononucleotides[29]. So far it has not been possible to estimate approximately the content of nucleoside polyphosphates in the s_2-fraction. Evidence that oligonucleotides are present in the latter fraction has been derived by fractionating s-RNA, which was isolated from pH 5 enzymes by means of starch block electrophoresis. As was pointed out in an earlier paper[23] soluble polynucleotides may thus be isolated which are essentially free of ATP. Chromatography of s-RNA obtained in this way on Ecteola also revealed the presence of the s_2-fraction.

Ultracentrifugal analysis of s_2- and s_3-RNA also suggested that the former fraction contains oligonucleotides. Prior to the sedimentation in the ultracentrifuge both fractions were dialysed to reduce their salt content. The low NaCl concentration of the s_2-fraction (0.07 *M*) permitted a shortening of the dialysis period and the isolation of a sufficient amount of this material. Table I shows the sedimentation constants at the concentrations of RNA and NaCl specified.

TABLE I

SEDIMENTATION BEHAVIOUR OF s_2- AND s_3-RNA IN THE ULTRACENTRIFUGE

Preparation	*RNA concentration mg/ml*	*NaCl concentration*	*Sedimentation constant*
s_2-RNA	3	0.1 *M*	4.00 S
s_3-RNA	8	0.2 *M*	4.08 S

The s_2-fraction contained 10–20 % of slowly sedimenting components, the main component showing a sedimentation constant which was of the order of that of s_3-RNA. This almost identical sedimentation of the s_2-component and s_3-RNA we are unable to explain but it is possible that the former is composed of oligonucleotides chelated by divalent ions. (s_2-RNA is also eluted from Ecteola by means of a 0.004 *M* EDTA solution in 0.01 *M* phosphate buffer.) The molecular weight of s_3-RNA is of the same order as that reported by others[2] for unfractionated s-RNA, *i.e.* 10–40,000.

Biochemical characterization of the s-RNA fractions

Labelling of s-RNA by amino acids: Following activation of amino acids by ATP and specific enzymes residing in the soluble fraction of the liver cell, the amino acids become attached to soluble RNA under the formation of 2′ or 3′ ester linkages at the terminal nucleotide[7–9]. Incubation of this s-RNA-amino acid complex with liver microsomes in the presence of ATP and GTP results in the incorporation of amino acid into microsomal ribonucleoprotein[2]. Obviously it is of interest to know which of the s-RNA fractions described above may function as a carrier of amino acids to the microsomes.

Hence the labelling of s-RNA by ^{14}C labelled amino acids was studied in a system containing pH 5 enzymes from rat liver, ATP and DL-[^{14}C]leucine or a ^{14}C labelled algal protein hydrolysate. This system was incubated in air at 37° for 10 min, cooled in crushed ice and extracted twice with 90 % phenol in water at 2–4°. RNA was precipitated with ethanol, redissolved in 0.01 *M* phosphate buffer and chromatographed on an Ecteola column (about 5 cm, diameter: 6.5 mm) at 2–4°. Eluate fractions of 3 ml were assayed for RNA and ^{14}C content. Radioactivity was corrected for absorption in NaCl and phosphate.

The results of a representative experiment with DL-[^{14}C]leucine are given in Fig. 2. Similar results were obtained in various experiments incubating a mixture of amino acids.

Fig. 2 shows that most of the radioactivity was recovered in the s_3-fraction. In two control experiments in which free [^{14}C]leucine and ^{14}C labelled algal protein

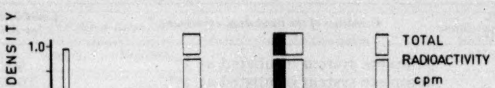

Fig. 2. Fractionation of s-RNA labelled with DL-[^{14}C]leucine on Ecteola. Incubation system: 10 ml pH 5 enzyme (280 mg protein, 3 mg s-RNA), 2 ml DL-[^{14}C]leucine (1.24 µmoles DL-leucine, 6.68 µC), 1 ml 0.01 *M* ATP. The shaded areas indicate radioactivity, the unshaded areas O.D. at 260 mµ.

hydrolysate were chromatographed in an identical fashion, all of the radioactivity was found in the s_1-fraction, suggesting that the s_1-RNA fraction of Fig. 2 did not carry amino acid but was contaminated with free amino acid. This was confirmed in the following way. Fraction 1 of Fig. 2 was concentrated by lyophilization and submitted to zone electrophoresis on starch. The radioactivity remained at the origin during the electrophoretic run. This means that fraction 1 contains no bound but free leucine only, as has been reported earlier[23]. In an electrophoretic experiment with a mixture of radioactive amino acids radioactivity could be detected in positions intermediate between the origin and the fast moving RNA zone, due to the presence of the acid amino acids. s_1-RNA, however, did not become labelled in an incubation experiment with pH 5 enzymes and a mixture of amino acids.

Fig. 2 reveals an additional radioactive zone eluted with 0.01 *M* phosphate buffer and appearing just before the s_2-RNA fraction. If the elution with 0.01 *M* phosphate buffer is not extended over a relatively large number of fractions, this second radioactive zone may coincide with the s_2-fraction. The non-identity of this zone with the s_2-fraction could also be established in experiments with a mixture of amino acids. The amount of radioactivity was always low and never exceeded 10 % of that recovered in the s_3-fraction. The same is true for the counts detected in the s_4-fraction eluted with alkali. So far no attempt has been made to identify these two minor fractions.

In conclusion it may be said that the s_3-fraction appeared to represent the biologically active carrier RNA in contrast to the s_2-fraction which was essentially devoid of radioactivity.

TABLE II

METABOLIC TRANSFER OF s-RNA TO m-RNA

Complete system: 1 ml liver microsome suspension containing 11.6 mg protein, 0.15 ml 105,000 × *g* liver supernatant containing 2.2 mg protein and 57 µg s-RNA (dialysed against 2 × 2 l 0.05 *M* Tris buffer pH 7.6, 0.025 *M* KCl, 0.005 *M* $MgCl_2$), 0.4 ml 0.05 *M* PEP, 0.3 ml 0.01 *M* ATP, 0.1 ml 0.006 *M* GTP, 0.1 ml pyruvate kinase (0.12 mg), 1.0 ml of a solution containing 0.15 mg s-[^{32}P]-RNA, total radioactivity 22,400 counts/min. Total volume 3.05 ml. Incubation in air at 37° for 10 min.

Expt. No.	*Condition of the incubation experiment*	*Counts/min/mg m-RNA*
1	Complete system incubated at 37°	900
2	Complete system incubated at 37°	1000
3	Complete system incubated at 0°	190
4	Complete system incubated at 0°	170
5	Complete system (37°) minus ATP, GTP, PEP, pyruvate kinase	150
6	Complete system (37°) minus ATP, GTP, PEP, pyruvate kinase	240
7	Complete system (37°) minus GTP	1150
8	Complete system (37°) minus GTP	1200

Metabolic transfer of s-RNA to m-RNA: In a previous communication a metabolic transfer of s-[^{32}P]RNA, labelled *in vivo*, to microsomal RNA was established using a system containing a 15,000 × *g* liver supernatant, ATP and 3-phosphoglyceric acid[15].

Similar results are obtained with isolated microsomes supplemented with soluble enzymes, ATP, GTP and a nucleoside triphosphate generating system (Table II).

Hultin and Von der Decken[10,11] observed a considerable reduction of the s-RNA transfer in the absence of GTP. Similar results were reported by Hoagland *et al.*[12–14]. In our system such a partial GTP requirement could not be demonstrated. No attempt was made to confirm the observations of these authors by more extensive washing of the microsomes.

In order to correlate the biochemical functions of s-RNA, subfractions of s-[^{32}P]RNA were isolated by means of chromatography on Ecteola as described above. The labelled s-RNA, which was isolated by phenol extraction from a 105,000 × *g* liver supernatant, was not dialysed but precipitated once with ethanol, redissolved in 0.01 *M* phosphate buffer and fractionated on Ecteola.

The s_3-fraction was dialysed against cold distilled water until the NaCl content was sufficiently reduced and was subsequently concentrated by lyophilization. The s_1- and s_2-fractions were lyophilized without prior dialysis, whereas the s_4-fraction was discarded.

All three polynucleotides were incubated at two concentrations (0.2 mg and 0.4 mg s-RNA per incubation flask) in the presence and in the absence of ATP, GTP, PEP and pyruvate kinase in a system containing microsomes and soluble enzymes.

As may be seen from Table III, s_3-RNA is the only polynucleotide of the three studied which could be transferred to m-RNA, although this RNA contained much less radioactivity than s_1- and s_2-RNA (see caption Table III). Consequently a rather high efficiency (about 17 %) of the transfer reaction may be calculated for Expt. 8. No transfer was observed in the absence of ATP, GTP, PEP and pyruvate kinase. GTP dependence was not studied in these experiments.

TABLE III

METABOLIC TRANSFER OF s-RNA SUBFRACTIONS TO m-RNA

Complete system: 1 ml liver microsome suspension containing 19 mg protein and 1.3 mg m-RNA, 0.15 ml 105,000 × *g* liver supernatant dialysed against 2 × 2 l Tris buffer of pH 7.4, containing 2.2 mg protein and 57 µg s-RNA, s-[^{32}P]RNA as specified. A: 0.3 ml 0.01 *M* ATP, 0.4 ml 0.05 *M* PEP, 0.1 ml 0.006 *M* GTP, 0.1 ml pyruvate kinase, 0.12 mg. Total volume 3.05 ml. Incubation at 37° in air for 10 min. Radioactivity s_1-RNA: 2,610,000 counts/min/mg; s_2-RNA: 85,500 counts/min/mg; s_3-RNA: 28,500 counts/min/mg.

Expts.	*s-[^{32}P]RNA incubated*		*Conditions of the incubation*	*Counts/min/mg m-RNA isolated*
	mg	*Fraction*		
1	0.2	s_1-RNA	Complete system	58
2	0.4	s_1-RNA	Complete system	52
3	0.2	s_1-RNA	Complete system minus A	15
4	0.4	s_1-RNA	Complete system minus A	38
5	0.2	s_2-RNA	Complete system	57
6	0.4	s_2-RNA	Complete system	71
7	0.14	s_2-RNA	Complete system minus A	19
8	0.2	s_3-RNA	Complete system	750
9	0.4	s_3-RNA	Complete system	950
10	0.2	s_3-RNA	Complete system minus A	59
11	0.4	s_3-RNA	Complete system minus A	51

DISCUSSION

So far only preliminary reports on the fractionation of soluble polynucleotides have appeared in the literature although more detailed communications have been announced. SMITH, CORDES AND SCHWEET[30] primarily dealt with the separation of s-RNA fractions showing an increased specificity for tyrosine and leucine respectively. HOLLEY *et al.*[31, 32] separated soluble polynucleotides by counter current distribution specific for alanine and tyrosine. GOLDTHWAIT[33] got two fractions by chromatography on Ecteola, showing similar sedimentation and base composition. Both fractions were labelled with [^{14}C]leucine to about the same extent although they showed a significant difference in incorporation of [^{32}P]AMP. A comparison of these results (see also [34]) with those of the present communication will have to await more information about the experimental details. The same holds true for a report by HOAGLAND *et al.*[2] concerning a similar fractionation of [^{14}C]leucine–s-RNA. The latter authors recovered most of their radioactivity in a fraction which they eluted from Ecteola by alkali.

Our investigations show that a polynucleotide fraction (s_3-RNA), which behaved homogeneously on Ecteola and during sedimentation in the ultracentrifuge, is involved in both amino acid- and polynucleotide transfer to the microsomal template. This fraction probably is still rather complex as SCHWEET *et al.*[30] and HOLLEY *et al.*[31, 32] demonstrated that different amino acids are carried by different polynucleotides. Assuming that the biologically active s_3-fraction, which has a molecular weight of 10–40,000, is not contaminated by smaller poly- or oligonucleotides, it is evident that polynucleotides consisting of at least 40–130 nucleotides are involved in both transfer reactions. Therefore it is of particular interest to look for possible intermediates of smaller sizes.

A search for intermediates was made in *in vitro* experiments in which s_3-[^{32}P]RNA was incubated in the presence of microsomes, soluble enzymes and cofactors. Following incubation the microsomes were sedimented and the soluble polynucleotides were extracted with phenol, not precipitated by ethanol, and fractionated on Ecteola. These experiments did not reveal any other radioactive polynucleotide than the s_3-RNA started with. It is possible that highly active intermediates may not accumulate to any extent during the incubation as long as microsomes are present. When the experiments were repeated, however, with a system containing soluble enzymes only, they did not show the formation of smaller polynucleotides either.

It may be concluded from these experiments that the enzymes, which eventually catalyse the production of polynucleotide intermediates, either reside in the membranes of the endoplasmic reticulum or in the RNP-particles itself.

Finally, it still remains a matter of speculation whether the two processes: amino acid- and polynucleotide transfer, which may be followed independently, are two manifestations of one mechanism. The GTP requirement of both, as reported by HULTIN, HOAGLAND AND ZAMECNIK and their coworkers[10–14], suggests a coupling of the two reactions. These authors used for their transfer experiments s-RNA, which was dialysed extensively and consequently may have been free of s_1- and s_2- but not of s_4-RNA. The fact that the two biochemical functions are confined to the polynucleotides residing in one fraction (s_3-RNA) strengthens the hypothesis that one mechanism is involved although the apparent complexity of the latter fraction[30–32] does not allow any definite conclusion so far.

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STUDIES ON CYTOPLASMIC RIBONUCLEIC ACID FROM RAT LIVER

II. FRACTIONATION AND FUNCTION OF MICROSOMAL RIBONUCLEIC ACID

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SUMMARY

1. Rat-liver microsomal RNA isolated by phenol extraction from deoxycholate-insoluble particles was fractionated on the resin Ecteola, yielding at least three fractions.

2. Following *in vitro* incubation of microsomes with ^{32}P labelled soluble polynucleotides, ATP, GTP, and a nucleoside triphosphate regenerating system, only one microsomal RNA fraction became radioactive.

3. This selective metabolic transfer of soluble RNA (or a derivative thereof) resulted in the attachment to microsomal RNA by covalent bonds.

4. Ribonuclease activity in the ribonucleoprotein particles became manifest after exposure of the particles to 8 *M* urea. The presence of this enzyme is discussed in connection with the transfer reaction.

INTRODUCTION

For a number of years circumstantial evidence has accumulated which indicates that ribonucleoprotein particles present in a variety of cells differing as much as those of liver and pancreas, plants and micro-organisms, are implicated in protein biosynthesis[1]. For a better understanding of the mechanism of the latter process a deeper insight in the structure of these particles is necessary.

Ultracentrifugal analysis revealed the presence of smaller subunits still consisting of about equal parts of RNA and protein. These two parts did not separate during electrophoresis in most cases[2-6] suggesting that the particles are composed of ribonucleoproteins of smaller sizes. The latter are probably cemented together through linkage of divalent cations. Removal of these cations by chelating agents or by other procedures promote a dissociation of the particles. In the present paper fractionation

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Abbreviations used: s-RNA, soluble ribonucleic acid; m-RNA, microsomal ribonucleic acid; RNP, ribonucleoprotein; AMP, adenosine monophosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetate.

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studies of the ribonucleic acids isolated from rat-liver microsomal particles will be described. Special attention is given to the question which fraction functions as an acceptor in the metabolic transfer of soluble polynucleotides to m-RNA[7–12].

MATERIALS AND METHODS

Liver microsomes were isolated from the same strain of rats in an identical fashion as described in the preceding paper[7]. The phenol extraction of RNA and the chromatography and electrophoresis procedures were also essentially identical to those reported there.

RESULTS

Fractionation of m-RNA on Ecteola

The insoluble ribonucleoprotein particles sedimented at 105,000 × *g* from a rat-liver microsomal fraction treated with deoxycholate[13] were extracted by phenol. This extraction procedure[14] offers the advantage of inhibiting ribonuclease and yielding a highly polymerized product[15]. Fig. 1 illustrates the fractionation of such a phenol extract on Ecteola. Three fractions were obtained, designated m_1-, m_2-, and m_3-RNA, respectively. m_1-RNA runs through the column in 0.01 *M* phosphate, m_2-RNA is isolated by applying a gradient elution with NaCl and EDTA, and m_3-RNA is removed from the column with 0.1 or 1 *N* NaOH. The latter fraction is always contaminated to some extent by unknown products from Ecteola which hampers the estimation of the m_3-RNA content. When m-RNA labelled *in vivo* with ^{32}P was isolated and fractionated, the results summarized in Table I were obtained. From these data it was

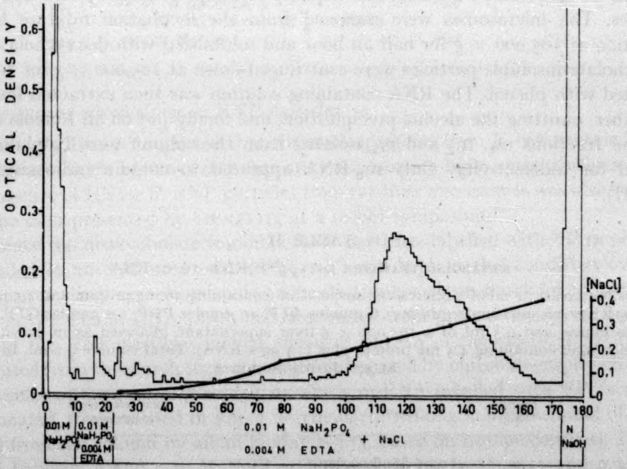

Fig. 1. Fractionation on Ecteola of m-RNA isolated by phenol extraction from RNP particles. O.D. was recorded at 260 mμ. NaCl concentrations are indicated by a concentration curve. Identical results were obtained in the absence of EDTA.

concluded that the amounts of m_2- and m_3-RNA are of about the same order of magnitude. The latter fraction may be resolved on Ecteola in two subfractions, being subsequently eluted with 0.1 *N* NH_4OH and 0.1 *N* NaOH.

Strictly identical results were obtained in experiments in which EDTA was omitted from the eluents (see also below). None of the fractions m_1, m_2 and m_3 was dialysable.

TABLE I

RADIOACTIVITY AND O.D. OF m-RNA FRACTIONS ISOLATED BY ECTEOLA CHROMATOGRAPHY*

Fraction No.	*Eluent*	*Total radioactivity counts/min*	*Total O.D. at 260 mμ***
m_1	0.01 *M* phosphate	670,000	189
m_2	0.01 *M* phosphate + 0.4 *M* NaCl	99,100	28
m_3	*N* NaOH	120,000	63

* Chromatography was performed without NaCl gradient but with the eluents specified.
** The O.D. read at 260 mμ was multiplied by the volume of the fractions and the products so obtained were enumerated.

The metabolic transfer of s_3-RNA to m-RNA

The metabolic transfer of s-RNA to m-RNA was shown in the preceding paper[7] to be confined to one of three s-RNA fractions studied, namely s_3-RNA. Hence it was of interest to determine which of the three m-RNA fractions may function as acceptor in this transfer reaction. The following experiment was therefore performed: a microsome suspension was incubated with s_3-RNA labelled *in vivo* with ^{32}P in the presence of ATP, GTP, a nucleoside triphosphate regenerating system and soluble enzymes. The microsomes were removed from the incubation mixture by sedimentation at 105,000 × *g* for half an hour and solubilized with deoxycholate[13]. The deoxycholate-insoluble particles were centrifuged down at 105,000 × *g* for 1.5 h and extracted with phenol. The RNA-containing solution was then extracted four times with ether, omitting the alcohol precipitation, and finally put on an Ecteola column.

The fractions m_1, m_2 and m_3 isolated from the column were lyophilized and assayed for radioactivity. Only m_3-RNA appeared to contain radioactivity (see Table II).

TABLE II

METABOLIC TRANSFER OF s_3-[^{32}P]RNA TO m-RNA

Incubation mixture: 2 ml of a microsome suspension (containing 38 mg protein and 2.5 mg RNA) 1.2 mg s_3-RNA (28,500 counts/min/mg), 6 μmoles ATP, 40 μmoles PEP, 1.2 μmoles GTP, 0.24 mg pyruvate kinase and 0.3 ml of a 105,000 × *g* liver supernatant (dialysed as in previous communication[7] and containing 4.4 mg protein plus 114 μg s-RNA). Total volume 6.1 ml. Incubation at 37° during 10 min.

Fraction No.	*Eluent*	*Total radioactivity counts/min*
m_1	0.01 *M* phosphate	4
m_2	0.01 *M* phosphate + 0.4 *M* NaCl	0
m_3	0.1 *N* NaOH	186

Fraction m_2-RNA, which was completely devoid of radioactivity, emerged from an Ecteola column under similar conditions as s_3-RNA[7]. Hence this soluble polynucleotide or a derivative thereof became so firmly attached to m_3-RNA that it did not dissociate under the conditions of phenol extraction and salt elution from the exchanger. This attachment also sustained exposure to a solution of 8 *M* urea and 0.004 *M* EDTA, as is shown by the following experiment.

After the incubation of the microsome suspension with s_3-[^{32}P]RNA and the necessary cofactors, a phenol extract of m-RNA was prepared as above and divided into two equal parts. One part was chromatographed as such on Ecteola, whereas the other was supplemented with urea and EDTA to a final concentration of 8 *M* and 0.004 *M*, respectively. The resulting RNA solution was put on Ecteola and eluted with the usual eluents which now included urea and EDTA in the specified concentrations. Table III illustrates that also under these conditions no other fraction than m_3-RNA appeared to be labelled, and it may be concluded that s_3-RNA or its derivative becomes covalently linked to m-RNA during the transfer reaction.

TABLE III

METABOLIC TRANSFER OF s_3-RNA TO m-RNA

A: represents a phenol extract of the incubation mixture (see Table II) which was chromatographed on Ecteola in the ordinary fashion. B: represents an identical aliquot of the phenol extract supplemented with 8 *M* urea and 0.004 *M* EDTA washed from the Ecteola column with the eluents specified which included however 8 *M* urea and 0.004 *M* EDTA.

Fraction No.	*Eluent*	*Total radioactivity* *A* *counts/min*	*Total radioactivity* *B* *(+ urea + EDTA) counts/min*
m_1	0.01 *M* phosphate	22	0
m_2	0.4 *M* NaCl in 0.01 *M* phosphate	0	7
m_3	0.1 *N* NaOH	223	194

The presence of ribonuclease in RNP-particles

Recently ELSON[16] reported that ribonucleoproteins from *E. coli* contain a latent ribonuclease which becomes manifest after exposure to high concentrations of urea. The presence of RNase in RNP particles from rat-liver microsomes was also suggested by some data presented by SIEKEVITZ at a recent symposium[17].

Hence the deoxycholate insoluble RNP particles, labelled with ^{32}P *in vivo*, were suspended in an 8 *M* urea solution at 2–4° and kept in this condition overnight. Subsequently they were submitted to zone electrophoresis on starch for 16 h (4.5 V/cm, 10–12 mA)[18]. The m-RNA which had dissociated from protein during the urea treatment of the RNP particles moved ahead of protein in the electrophoretic run. It was eluted from the starch segments and fractionated by chromatography on Ecteola. The results of such an experiment in which m-RNA labelled with ^{32}P *in vivo* was fractionated are presented in Fig. 2. Particularly striking is the low yield of m-RNA eluted from the column by alkali (about 2–3 %, based on isotope content). The RNA eluted between 0 and 0.10 *M* NaCl and that eluted between 0.17 and 0.3 *M* NaCl was dialysed against cold distilled water, lyophilized and analysed in the analytical ultracentrifuge. As may be concluded from Table IV both fractions represent RNA of relatively low molecular weight. The former appeared to be polydispers, showing an average sedimentation constant of 0.83 S. When the ARCHIBALD procedure[19] is

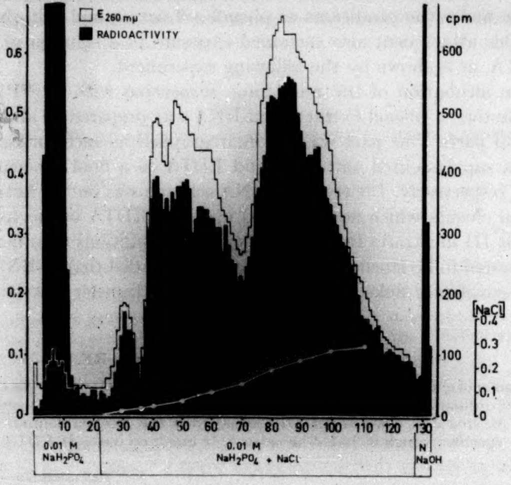

Fig. 2. Fractionation on Ecteola of m-RNA isolated by starch block electrophoresis of urea-treated RNP particles. In order to make an approximate estimation of the amount of RNA eluted with alkali, m-RNA was labelled *in vivo* with ^{32}P. The black areas represent radioactivity, the unshaded areas O.D. recorded at 260 mμ. The increasing NaCl concentrations are indicated by a concentration curve.

TABLE IV

SEDIMENTATION OF TWO m-RNA FRACTIONS OBTAINED BY ECTEOLA CHROMATOGRAPHY

m-RNA eluted between	*RNA concentration (mg/ml)*	*NaCl concentration*	*Sedimentation constant*
0–0.10 *M* NaCl	3	0.1 *M*	0.83 S
0.17–0.30 *M* NaCl	8	0.2 *M*	2.53 S

applied, an average molecular weight of 3100 may be calculated for these polynucleotides.

These data demonstrate clearly that a high proportion of the starting material was degraded during the isolation procedure.

EDTA treatment

In a duplicate experiment in which m-RNA was isolated under strictly identical conditions as those of Fig. 2 (isolated during the same electrophoretic run) the m-RNA was supplemented with 0.004 *M* EDTA before chromatography on Ecteola (Fig. 3). The total recovery in this experiment was the same as that obtained in Fig. 2. Due to the presence of EDTA, however, the amount of material isolated in the absence of NaCl increased from 1.2 % to 22 %. When the EDTA was temporarily omitted from the 0.01 M phosphate eluent the removal of these polynucleotides immediately stopped but resumed as soon as EDTA was included again in the eluent.

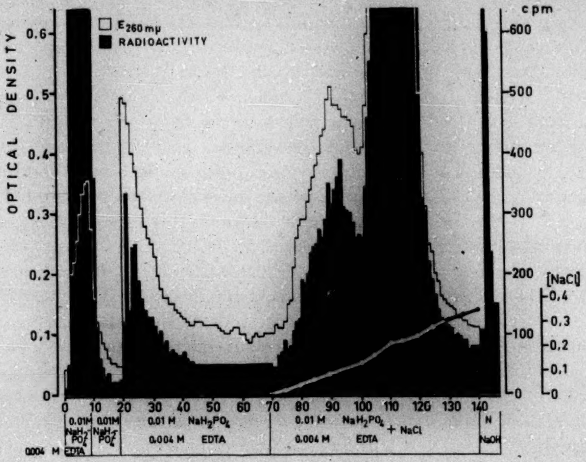

Fig. 3. The effect of EDTA on the fractionation of m-RNA on Ecteola. m-[^{32}P]RNA was identical to that studied in Fig. 2. The same amount was submitted to fractionation. EDTA was included in the eluents when indicated. Black areas represent radioactivity, unshaded areas O.D. recorded at 260 mμ. The increasing NaCl concentrations are indicated by a concentration curve. The recovery of RNA eluted between 0.13 and 0.3 M NaCl was the same as that of the corresponding fractions of Fig. 2. The presence of EDTA, however, causes a sharpening of the zones[7].

DISCUSSION

The present investigations show that microsomal RNA isolated by phenol extraction from deoxycholate-insoluble particles of rat-liver cytoplasm can be resolved in at least three fractions. The fractions listed in Table I were not analysed separately in the ultracentrifuge due to the fact that degradation during alkaline elution is almost inevitable. Some progress has recently been made in this laboratory to work out a different fractionation procedure for m-RNA. It is hoped to present a more detailed ultracentrifugal analysis of m-RNA components in a forthcoming paper.

When the unfractionated phenol extract was analysed in the ultracentrifuge two components were visible in the sedimentation diagram, one with a sedimentation constant of 19 S, the other with one of 12 S (in 0.01 M Tris buffer, pH 7.4). Under somewhat modified conditions (the phenol extract had been submitted to zone electrophoresis on starch) two peaks were observed of 11.6 S and 7.6 S, respectively (again in 0.01 M Tris buffer of pH 7.4).

Recently HALL AND DOTY[20] reported that microsomal RNA isolated from calf liver consist, of two components having sedimentation constants of 28 and 18 S to which they assigned molecular weights of about 1,300,000 and 600,000 respectively. They concluded from experiments in which their RNA preparations were heated to 85° that these RNA components consist of subunits showing sedimentation constants of about 8.2 S (molecular weight of 120,000). The dissociation of microsomal RNA into subunits appeared to involve the breaking of internucleotide hydrogen bonds.

The results of GIERER[21], obtained with RNA extracted from rat-liver microsomes, non-treated with deoxycholate, revealed the presence of two main components having molecular weights around 1,800,000 and 600,000, respectively.

From the present studies it may be concluded that our m-RNA preparations contain at least two RNA's of high molecular weight which tentatively may be identified as composing the m_3-fraction (polyadenylic acid preparations of molecular weight 270,000 and 400,000 respectively also appeared in this fraction on Ecteola chromatography). The chromatographic behaviour of the latter is unaltered by treatment with EDTA. In addition it has been shown in this paper that aggregates of smaller polynucleotides, if they are present, do dissociate in the presence of the chelating agent (compare Fig. 3). Hence, deaggregation of m_3-RNA if occurring by exposure to EDTA is not revealed by Ecteola fractionation. The effect of heating in the chromatographic pattern has not been studied. The heterogeneous nature of m_3-RNA appears from the resolution in two sub-fractions, one being eluted with 0.1 N NH_4OH, the other with 0.1 N NaOH.

Particularly interesting is the finding that only m_3-RNA is able to act as an acceptor of label in the metabolic transfer reaction in which soluble polynucleotides (s-RNA) are involved. Such a selective labelling of only the high molecular weight fractions of m-RNA excludes the possibility that m_2-RNA is a degradation product of m-RNA arising during the phenol extraction procedure. The elucidation of the metabolic role of m_2-RNA has to await further experimentation.

The present experiments further showed that the soluble polynucleotide s_3-RNA or a derivative thereof became attached to microsomal RNA by bonds which are covalent in nature. The mechanism of this transfer reaction is still essentially obscure and the search for possible intermediates has so far not met with success (compare preceding paper). If it is assumed that poly- or oligonucleotides of smaller size are really involved in this reaction, the intermediate formation of these compounds does not seem to depend on the presence of soluble enzymes residing in the soluble part of the cell[7]. Consequently they have to be looked for either in the microsomal membranes or in the RNP particles. The presence of RNase in the RNP particles may present a serious complication in studies of this type, although a specific role of this microsomal RNase in connection with the polynucleotide transfer reaction is not excluded.

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EFFECT OF IRRADIATION ON CELL DIVISION AND NUCLEIC ACID SYNTHESIS IN STRAIN U-12 FIBROBLASTS[*]

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SUMMARY

The sequence of events following an irradiation of 500 R was investigated in strain U-12 fibroblasts. The irradiation caused a rapid and almost complete inhibition of cell multiplication, as measured by nuclear counts, the mitotic index, and the number of colonies produced from single cells. Although mitotic figures reappeared and increased in frequency to abnormally high levels 13–20 h after irradiation, a recovery in cell multiplication did not occur. Time-lapse cinematography showed that the increased incidence in mitotic figures was very probably caused by the increased time spent by these cells in abortive mitoses. In contrast to the immediate inhibition of mitosis, DNA synthesis was reduced at a later time, falling to less than 50% of the control 18–24 h after irradiation. In this system, therefore, the immediate inhibition of mitosis cannot be the result of a deficiency in DNA synthesis, as measured by the number of individual cells incorporating [^{3}H]thymidine or by the specific activity of DNA after incorporation of ^{32}P. On the other hand, it is possible that the reduction in the number of cells synthesizing DNA results entirely from the inhibition of mitosis and subsequent failure of the orderly progression of cells through the mitotic cycle.

Abbreviations used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

[*] A preliminary report of these experiments has appeared previously[1].

INTRODUCTION

The role of deoxyribonucleic acid (DNA) in radiation injury has been a subject of interest for many years, but still remains to be completely elucidated. In some cases an inhibition or delay of DNA synthesis seems to be an early and perhaps primary effect of irradiation[2–5], whereas in other systems inhibition of DNA synthesis is thought to be a secondary effect resulting from an inhibition of cell division or a change in the cell population of the tissue investigated[6]. The use of tissue culture systems circumvents some of the difficulties caused by changing cell populations in *in vivo* studies. In general, very small amounts of irradiation (50 R) have been found to inhibit mitosis in mammalian cells *in vitro*, whereas very high doses (100,000 R or higher) are required for immediate lethal effects[7]. In Strain-L fibroblasts, cell division has been found to be inhibited by irradiation, but no specific inhibition of DNA synthesis was evident as measured by the amount of DNA per cell or by incorporation of labeled precursors[8–10]. The use of autoradiography permits the study of individual cells at various stages in the cell cycle at intervals after irradiation. Using this technique, LAJTHA found that 150 R was sufficient to reduce the number of bone marrow cells entering the period of DNA synthesis by about 50 %, while more than 500 R was necessary to inhibit cells already synthesizing DNA at the time of irradiation[3]. In contrast, PAINTER AND ROBERTSON observed an increase in the number of HeLa cells synthesizing DNA 6 h after the administration of 500 R, indicating that the cells might be delayed in the period of DNA synthesis[11]. In the present study, the sequence of events following an irradiation of 500 R was investigated in strain U-12 fibroblasts. The irradiation was sufficient to cause a prolonged and profound inhibition of cell multiplication. However, DNA synthesis, as measured by incorporation of labeled precursors, was not reduced immediately, the maximum inhibition occurring 18–24 h after irradiation.

MATERIALS AND METHODS

Cells

Strain U-12 fibroblasts, originally derived from human uterus[12], were obtained from Dr. H. E. SWIM. The cells were grown on glass in T-flasks[13], or on coverslips placed in Leighton tubes. The chemically defined medium, S-103[14], was supplemented with 20 % horse serum. Cells were subcultured weekly, and the medium was replaced on the third and fifth days thereafter. Growth of the cells was determined by the nuclear counting method of SANFORD *et al.*[15]. The ability of single cells to yield colonies according to the method of PUCK *et al.*[16], was also used as an index of irradiation effect. About 200 cells were plated on a feeder layer previously irradiated with 2000 R. The plated cells were then irradiated with 50–500 R and the number of macroscopic colonies present 18 days later were counted and compared to similarly prepared unirradiated plates.

Irradiation

On the second or third day after subculture the cells were irradiated through the glass surface of T-flasks or test tubes. The dose was measured with a Bomke instantaneous dosimeter placed inside an empty flask. The radiation was given at the rate of 100–126 R/min (220 kV, 15mA, filtered through 0.5 mm Cu and 1 mm Al). The medium was replaced immediately after irradiation. In all cases a series of non-irradiated control cultures of similar age were prepared and carried along through all the subsequent analyses.

Incorporation of ^{32}*P*

Radioactive phosphate* was added to the medium at intervals after irradiation, to give a final concentration of 0.2 μC/ml. The concentration of phosphorus in the medium was 0.0008 *M*. 3 or 6 h after the addition of the ^{32}P the cells were harvested, and nucleic acids were extracted and separated by the SCHMIDT-THANNHAUSER technique[17]. The cells from 4 T-60 flasks were combined for each extraction, and duplicate extractions for control and irradiated cells were carried out at each time interval. DNA and RNA were measured by their absorption at 260 mμ, and by colorimetric assays for pentose[18], and deoxypentose[19]. Duplicate aliquots of the RNA and DNA fractions were plated on stainless steel planchets and assayed for ^{32}P with an end-window Geiger-Mueller counter.

Incorporation of [4-^{3}H]*thymidine into DNA*

Cells were grown on cover slips in Leighton tubes. Following irradiation the incorporation of [^{3}H]thymidine** was studied for consecutive 6-h periods. In each case the precursor was added to give a final concentration of 0.05 μC/ml. Carrier thymidine was added to the labeled material to provide a higher concentration of the precursor and to reduce radio-toxicity. The final concentration of thymidine in the medium was 0.01 μmoles/ml. After a 6-h incorporation period, the coverslips were rinsed in 0.9% saline, fixed in anhydrous methanol, and mounted on gelatinized microscope slides. Stripping film[20] (Kodak permeable base) or liquid emulsion[21] (Kodak NTB 3) was applied and, after an exposure of 1–2 weeks at 4°, the film was developed and the cells stained with Harris hematoxylin. The number of positive cells in a total of 2000 cells on each slide was determined at each interval.

Incorporation of [^{3}H]*cytidine into RNA*

Incorporation of [^{3}H]cytidine** was determined as described for [^{3}H]thymidine, the final concentration of this precursor also being 0.05 μC/0.01 μmoles/ml. After the cells were fixed in methanol, they were treated with a 0.01% solution of deoxyribonuclease*** for 2 h at pH 6 and 37° in the presence of 0.005 *M* $MgSO_4$[22]. It had previously been ascertained that this treatment removed all DNA from the cells, as evidenced by the complete removal of labeling from cells exposed to [^{3}H]thymidine. After enzymic treatment, the coverslips were rinsed in distilled water, and film was applied, exposed, and developed as described earlier. The cells were then stained, and the number of cells with positive nuclei in a total of 1000 cells was determined for each slide.

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* Obtained as $H_3{}^{32}PO_4$ from Oak Ridge National Laboratory and neutralized with NaOH before addition to the medium.

** Obtained from Schwarz Laboratories, Inc., Mount Vernon, N.Y.

*** 1 × recrystallized enzyme, obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Mitotic index

Cells were grown on coverslips in Leighton tubes as described above. At various intervals after irradiation coverslips were removed from irradiated tubes, as well as from 2–4 controls, and were rinsed in saline, fixed in methanol, and stained with Harris hematoxylin. The number of mitotic figures in a total of 10,000 cells was determined on each slide, and the mitotic index, expressed as mitotic figures per 1000 cells, was determined.

RESULTS

Effect of irradiation on multiplication of strain U-12 fibroblasts

The cells in 3 or more T-flasks were harvested at daily intervals after exposure to 125–500 R and were counted by the nuclear counting procedure[15]. As shown in Fig. 1, the onset of logarithmic growth occurred 1 day later in cultures irradiated with 125 R than in the control cultures. Doses of 250 R and 375 R produced a progressively greater inhibition of cell multiplication, and after 500 R the inhibition was almost complete. However, very little actual cell death occurred, as indicated by the uptake of eosin Y[23]. The length of the lag phase in control cultures varied from week to week, but the average growth constant for logarithmically growing cells in a total of 12 experiments was 0.55 ± 0.05/day, corresponding to a doubling time of approx. 30 h.

Irradiation also affected colony production by single cells, 500 R reducing the number of colonies to 3 % of the number obtained with unirradiated plates (Fig. 2).

An irradiation of 500 R had a pronounced effect on the mitotic index. Thus, within 105 min after irradiation, the mitotic index was only 5 % of the control (Fig. 3). It remained at a very low level for approx. 13 h and then increased sharply, until at

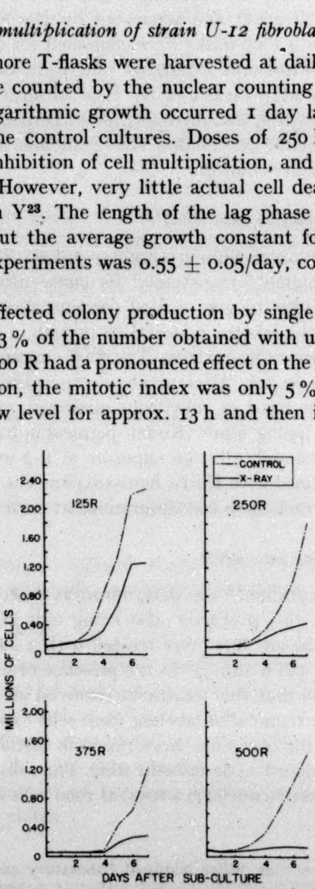

Fig. 1. Effect of irradiation on multiplication of U-12 cells. The nuclei from replicate control and irradiated flasks were counted at daily intervals. Radiation was given on the second day after subculture.

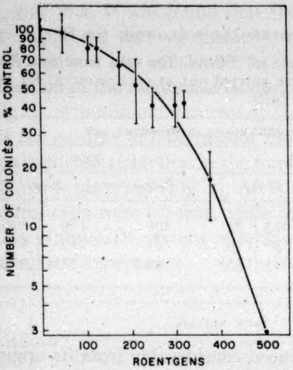

Fig. 2. Effect of irradiation on colony production by single U-12 cells. 200 cells were plated on a feeder layer previously irradiated with 2000 R. 4–8 flasks containing the newly plated cells were irradiated at each dose level indicated above, and visible colonies were counted after 18 days and compared to similarly treated controls. Vertical lines at each point represent the standard deviation.

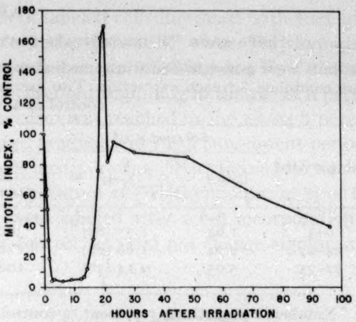

Fig. 3. Effect of 500 R on the mitotic index. The number of mitotic figures in a total of 10,000–100,000 cells were counted at each interval for both control and irradiated cells. The overall average mitotic index for control cells was 25.3 mitoses per 1000 cells.

19–20 h the number of mitotic figures was considerably above the normal value. A decrease then occurred, and by 4 days after irradiation the level had dropped to 40% of the control. Although the sudden reappearance of mitotic figures 13 h after irradiation might be indicative of a recovery, there was no return of cell multiplication as determined by nuclear counts and colony production (Fig. 1, 2). When the mitotic figures appearing 18–20 h after irradiation were examined in a living culture by means of time-lapse cinematography*, it was seen that these cells remained in mitosis for very long periods and that few actually accomplished a successful division. In a preliminary study of 13 individual mitoses occurring 13 h or more after the administration of 500 R, the average duration of mitosis was 12 h and the time ranged from 2–38 h. None of the 13 mitotic figures resulted in the production of 2 daughter cells. In unirradiated cells, the average mitotic time was slightly less than 1 h, as measured by direct observations with time-lapse cinematography as well as by calculations based on the mitotic index, which averaged 25.3 in 7 experiments in which a total of approximately 10^6 control cells was counted.

Incorporation of ^{32}P

Initially, incorporation of ^{32}P was carried out for 3-h periods at daily intervals following 500 R. In one experiment (Table I), there was a slight decrease in the specific activity of the DNA when the incorporation was carried out immediately after irradiation, but the significance of the reduction was questionable. The specific activity was reduced to 60% of the control value 24–27 h after irradiation, but at 72–75 h was again equal to the control value. However, at 72–75 h, the specific

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* Time-lapse cinematography was carried out in conjunction with the Physics Section, Western Reserve University Atomic Energy Medical Research Project.

TABLE I

THREE-HOUR ^{32}P INCORPORATION AT DAILY INTERVALS FOLLOWING 500 R

The cells were grown in T60's in a medium containing 0.2 μC ^{32}P/ml. The cells from four flasks were combined for each extraction. Two extractions were carried out at each interval for both control and irradiated cells.

Hours after X-*ray*	*Cell count* × *10*^{6}		*Specific activity, counts/min/u.v. unit*			
			RNA		*DNA*	
	Control	X-*ray*	*Control*	X-*ray*	*Control*	X-*ray*
0–3	1.64	1.35 (82)*	3093	2623 (85)*	206	169 (82)*
24–27	2.74	1.86 (68)	1288	1530 (119)	191	115 (60)
72–75	5.65	2.14 (38)	1085	1633 (155)	49.1	52.4 (106)

* Number in parenthesis represent % control.

activity of the DNA of the control cells had decreased considerably from its original value, perhaps because of crowding of the cells in the culture flask. The irradiation caused no marked reduction in the specific activity of the RNA. Since there appeared to be a decrease in DNA synthesis during the first 24 h following irradiation, incorporation of ^{32}P was carried out for consecutive 6-h periods during this time. Table II summarizes these experiments. Although irradiation caused no significant reduction in the incorporation of ^{32}P into DNA during the first 6 h following irradiation, a very slight but progressive decrease in the specific activity occurred in the 6–12 and 12–18 h periods, followed by a relatively sharp drop to a value 47 % of the control in the 18–24 h period. No significant change was noted in the specific activities of acid-soluble phosphorus or RNA. The specific activity of the DNA of the unirradiated cells did not change significantly in these experiments.

TABLE II

SIX-HOUR ^{32}P INCORPORATION AT INTERVALS FOLLOWING 500 R

The cells from 4 T60 flasks were combined for each extraction. Two separate extractions were carried out for control and for irradiated cells. Each number below represents the average value of 2 irradiated samples divided by the average value of 2 control samples.

Time of incorporation Hours after X-*ray*	*Specific activity: % Control*					
	Acid-soluble P		*RNA*		*DNA*	
0–6	117 92 108 136	113	121 108 87 77	97	145 102 93 68	102
6–12	113 97 102	104	156 95 123	125	85 93 82	87
12–18	95 90	92	95 104	100	72 83	78
18–24	102 87	95	69 97	83	52 42	47

Incorporation of [^{3}H]*thymidine and* [^{3}H]*cytidine*

In preliminary experiments[1], [^{3}H]thymidine was added to the medium immediately after irradiation, and the number of labeled cells was determined after 3, 6, 9, 24, and 30 h. It was found that the number of labeled cells increased with time at a rate equal to that of similarly treated controls. However, when $[^{3}H]$thymidine incorporation was measured for successive 6-h periods following 500 R, a definite reduction in the number of labeled cells was observed beginning in the 12–18 h period (Fig. 4). A minimum value of 27 % of the control was reached in the 18–24 h period, but the number of labeled cells then increased gradually in the 3 subsequent periods. No significant reduction in the number of cells incorporating $[^{3}H]$cytidine into nuclear RNA was observed (Fig. 4). After a 6-h incorporation of $[^{3}H]$thymidine, 47 % of the control cells were labeled, while 97–100 % were labeled after a 6-h incorporation of [3H]cytidine. The extent of labeling of the control cells did not change significantly during the experiment.

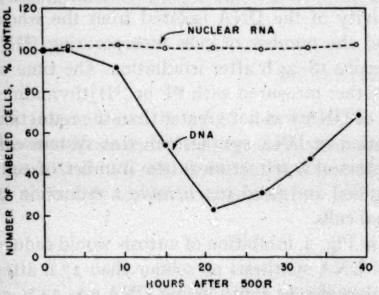

Fig. 4. Effect of 500 R on the number of cells synthesizing DNA and RNA. $[^{3}H]$thymidine or $[^{3}H]$cytidine was added immediately or at 6, 12, 18, 24, 30 or 36 h after 500 R. After a 6-h incorporation, the percentage of labeled cells was determined, counting a total of 1000–8000 cells at each interval. Points are plotted at the center of the 6-h interval. In 6 h 47 % of the control cells incorporated $[^{3}H]$thymidine into DNA, while 97 % incorporated $[^{3}H]$cytidine into nuclear RNA.

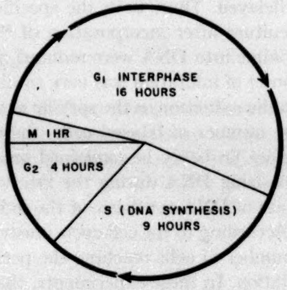

Fig. 5. Cell cycle of strain U-12 fibroblasts.

According to Howard and Pelc[24], the cell cycle can be considered to consist of the following periods in order: mitosis (M), interphase (G_1), DNA synthesis (S), interphase (G_2), mitosis, etc. The approximate durations of these periods in strain U-12 fibroblasts are shown in Fig. 5. The doubling time of 30 h and mitotic time of 1 h were determined as described above. Since 47 % of the control cells incorporated $[^{3}H]$thymidine in 6 h, the period of DNA synthesis was approximately 9 h, as calculated by the method of Painter and Robertson for cells in logarithmic growth[11]. The minimum time for the G_2 period may be estimated by the time necessary for the appearance of the first labeled mitotic figure. This time was 4 h in these experiments, but can apparently vary greatly from cell to cell in the same culture[25]. The maximum time for the G_1 interphase for strain U-12 fibroblasts was 16 h, as calculated by difference.

DISCUSSION

In these experiments an irradiation of 500 R caused an immediate and rapid drop in the incidence of mitotic figures (Fig. 3), so that within 105 min the level was only 5 % of the control. These results are in agreement with the concept that cells already in mitosis at the time of irradiation finish division, but that new cells are inhibited from entering mitosis[26]. In this system, the mitotic figures were absent for 13 h, but then reappeared and increased markedly, until at 19–20 h the mitotic index was considerably above the normal level. Observation of these cells with time-lapse cinematography showed that the mitotic interval was greatly prolonged and that very few cells actually succeeded in division. The prolonged duration of mitosis would cause an increase in the number of cells in mitosis at any instant, as is reflected in the abnormally high mitotic index 19 or more hours after irradiation. The discrepancy between the apparent recovery in mitosis and the lack of recovery shown by nuclear and colony counts is explained by the observation that the mitoses observed 13 h or more after irradiation were largely abortive.

In contrast to the immediate inhibition of mitosis, the reduction in DNA synthesis was delayed. Thus, both the specific activity of the DNA isolated from the whole cell culture after incorporation of ^{32}P and the number of cells incorporating [^{3}H]-thymidine into DNA were reduced maximally 18–24 h after irradiation. The time of the onset of inhibition was very similar whether measured with ^{32}P or [^{3}H]thymidine. Since the reduction in the specific activity of DNA was not greater than the reduction in the number of labeled cells, the reduction in DNA synthesis in this system can therefore probably be explained on the basis of a reduction in the number of cells synthesizing DNA during the interval studied and need not involve a reduction in the rate of DNA synthesis of the individual cells.

According to the cell cycle illustrated in Fig. 5, inhibition of mitosis would reduce the number of cells reaching the period of DNA synthesis no sooner than 17 h after irradiation. In these experiments, the number of cells synthesizing DNA was 54 % of the control in the 12–18 h period following irradiation, and 23 % of the control in the 18–24 h period. Although the reduction occurred somewhat earlier than would be expected if caused entirely by an inhibition of mitosis, it is possible that the G_1 period might be shorter than 16 h in some of the cells. LAJTHA *et al.* found that the number of bone marrow cells synthesizing DNA from [^{14}C]formate was reduced slightly 11–16 h after 135–500 R, and fell to 60 % of the control in the 17–22 and 22–27-h periods[3]. The time-course of the inhibition was very similar to that observed in the present investigation but the G_1 period of the bone-marrow cells was calculated to last 25–30 h[27], and the results were therefore interpreted as an indication of an inhibition of cells in the G_1 period at the time of irradiation. Preliminary results, however, have shown no evidence for a G_1 inhibition with strain U-12 fibroblasts. Thus when [^{3}H]thymidine was added immediately after irradiation and the progressive labeling was studied over the next 30 h, the number of labeled cells increased at the same rate in the irradiated cultures as in the controls. Neither was there any evidence of a delay of the cells in the period of DNA synthesis, as was observed in HeLa cells by PAINTER AND ROBERTSON[11]. The appearance of more labeled cells in the 30–36-h and 36–42-h periods after irradiation (Fig. 4) might result from synthesis of DNA by the cells which entered mitosis 13 h and later after irradiation, and which spent long periods in mitosis. Although these cells failed to divide, it is possible that they finally proceed through G_1 interphase into the period of DNA synthesis.

In contrast to the limited period of DNA synthesis, RNA appears to be synthesized continuously throughout the cell cycle. Thus, 97–100 % of the deoxyribonuclease-treated cells were labeled after a 6-h incorporation of [^{3}H]cytidine. A block in the cell cycle, although preventing cells from entering the period of DNA synthesis at the proper time, would not necessarily lead to a block in RNA synthesis. Thus, although the inhibition of DNA synthesis appears to be a specific and marked effect of irradiation in this system, it may possibly result entirely from the inhibition of mitosis and subsequent failure of the orderly progression of cells through the cell cycle. Although an independent effect on some early phase of DNA metabolism cannot be entirely excluded, it seems quite clear that the immediate inhibition of mitosis (Fig. 3) cannot be the result of an inhibition of DNA synthesis, as measured by the incorporation of precursors into DNA. Instead, the irradiation must affect cells which have already acquired a double complement of DNA and are ready to enter mitosis. The nature of the events occurring between completion of DNA synthesis and the onset of visible mitosis, and the effect of irradiation upon such events remain to be elucidated.

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SEPARATION OF BIOLOGICALLY ACTIVE COMPONENTS FROM SCORPION VENOMS BY ZONE ELECTROPHORESIS

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SUMMARY

Filter paper and starch electrophoresis were used for the separation of biological active components present in the venoms from the Brazilian scorpions *T. serrulatus* and *T. bahiensis*.

A toxic component (Scorpiotoxin T), a smooth muscle active substance (Scorpiokinin), a permeability factor and hyaluronidase were separated from the whole venom by these techniques.

Venoms from *T. serrulatus* and *T. bahiensis* are qualitatively very similar in chemical composition, but vary in toxicity and in the proportions of their active components. Venom collected from scorpions captured in different places in the same locality may show differences in chemical composition.

In contrast to snake venoms those from the scorpion are poor in enzymic activities. Among the enzymes studied hyaluronidase was the only one that showed considerable activity.

INTRODUCTION

In a previous paper[1] we have shown that the venom from Brazilian scorpions, *Tityus serrulatus* and *Tityus bahiensis*, have a complex biochemical constitution. The main toxic component was separated by filter paper electrophoresis techniques from the natural venom and from extracts of the telsons of the arachnids. Additional studies have, however, shown that these venoms contain several biologically active components. Further, it has been found that venom collected from batches of scorpions captured in different places exhibit differences in chemical composition as well as in biological activity. In this paper the results of these studies are presented.

METHODS

Venoms from the two most prevalent species of scorpions in Brazil, *T. serrulatus* and *T. bahiensis*, were used. The arachnids were caught in the Ribeirão Preto area and in the vicinity of Belo Horizonte, MG. They were classified and kept in separated cages in the laboratory. The venom was obtained by electric stimulation of the distal coils of the tail[2] and the drops which flowed from the telsons were dried in a vacuum dessicator and kept in the dark until they were used. Some experiments have been done with venoms from the Institute Butantã. These came from a venom pool obtained from scorpions collected in several places of the country*.

Zone electrophoresis was done with phosphate buffers at pH 7.4, ionic strength 0.1 or 0.05, 8–16 h in the cold room. Filter paper separations have been carried out according to the method of WIELAND AND FISCHER[3], using 0.5–1.0 mg of the dried venom dissolved in the buffer, the solution being applied on Whatman No. 1 paper strips, 2 cm wide. Starch electrophoresis was done in a plastic trough by the method described by KUNKEL AND SLATER[4], using 5–7 mg of venom. Paper strips were stained with amido black and a filter paper print was usually taken from the starch paste and stained in the same way. For the biological assays transverse segments 0.5 cm wide were cut out from the paper and eluted with 1.5 ml of saline solution; from the starch, blocks 1 cm wide were taken off and eluted with 5 ml water or saline.

The toxic activity was measured, using a 2+2 assay, by determination of the survival times of mice (18–20 g) injected intraperitoneally with aliquots of the venom eluates[5]. The contractile activity of the smooth muscle was assayed in a guinea pig ileum preparation perfused with Tyrode solution containing, in most of the experiments, 2.5 mg of morphine/l[6]. Whole venom was used as reference standard. Capillary permeability studies were done in rats weighing 60–75 g into the tail-veins of which 2 mg of Evans Blue dye dissolved in 0.5 ml saline was injected; 30 min after administration of the dye 0.02 ml of venom solutions or electrophoresis eluates were injected intradermally into the shaved abdominal skin or paws of the hind legs[7]. The results were read 30 min after the venom injection, when the rats were killed, the abdominal skin was removed and the injected areas were observed by transparency for dye extravasation. Phospholipase activity was measured by the method of NEUMAN AND HABERMAN[8] and hemolytic activity was tested with a 0.6 % rabbit red cell suspension. Hyaluronidase activity was carried out by the viscosity reduction method[9], proteolytic activity by ANSON's method and coagulation tests with a partially purified preparation of fibrinogen obtained by alcohol precipitation. The effect of trypsin and chymotrypsin on the active fractions was studied by incubation of the enzymes (crystallized, Armour) with lyophilized eluates from starch electrophoresis dissolved in appropriate buffers and the products were assayed in a guinea pig ileum preparation, desensitized for trypsin. Acetylcholinesterase activity was measured by AMMON's method[10] using Warburg's manometric techniques, as described by BIRKHAÜSER[11]. Inhibition of specific and unspecific acetylcholinesterase was measured by the method of ZELLER AND BIRKHAÜSER[12].

RESULTS

Zone electrophoresis

T. serrulatus and *T. bahiensis* venoms show a similar protein pattern in the electropherograms. The most frequent pattern obtained in the analysis of these venoms displays, at pH 7.4, four components which migrate to the cathode and two or three rather diffuse components moving to the anode. An apparent discrepancy of this model was observed in the analysis of a venom collected from a batch of scorpions captured at Ribeirão Preto in the years 1954–1955 and classified by its morphological characteristics as *T. serrulatus*. As we[1] have previously shown, filter paper electrophoresis of this venom, reveals at pH 7.4, a large protein component with a negative charge and only a small amount of positively charged material (Fig. 1).

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* The authors are grateful to Drs. W. BÜCHERL and L. A. RIBEIRO DO VALLE for these venoms.

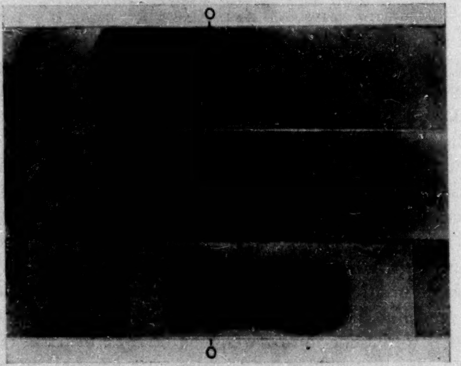

Fig. 1. Electropherograms of venoms from *T. bahiensis* (A) and *T. serrulatus* (B and C). The patterns shown in A and B are common and C is rare. The main toxic component moved 0.5–1.0 cm in the direction of the cathode in these venoms. (Phosphate buffer pH 7.4, $\mu = 0.1$, 16 h, 9 V/cm).

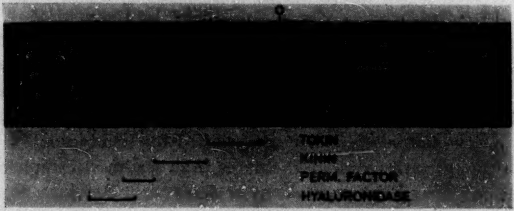

Fig. 2. Electropherogram of venom from *T. serrulatus*. (Phosphate buffer pH 7.4, $\mu = 0.05$, 14 V/cm). The figures show the localization of the active components in the paper as evidenced by the biological assay of eluates from corresponding transversal segments.

In the paper, the separation of biological active material was better achieved at pH 7.4 with a phosphate buffer of ionic strength 0.05. Because there was, with this diluted buffer, a larger migration of protein components in a shorter time, associated, in addition, with a slight modification of its relative mobilities, a clearer distinction of the various biological active materials was obtained (Fig. 2). Starch electrophoresis, however, allows the separation of larger amounts of venom, due to the electrosmotic flow, some spreading of the components was observed.

Smooth muscle contractile activity and toxicity

Assay of the venom from Brazilian scorpions on guinea pig ileum preparations determines a delayed contraction of the smooth muscle structure[1,6]. The activity of venom from *T. bahiensis* on smooth muscle is two or three times greater than that obtained from *T. serrulatus*. If bradykinin is used as a reference substance the assay in the guinea pig ileum preparation indicates for *T. bahiensis* an activity equivalent to 20–25 units of bradykinin/mg of dried venom and for *T. serrulatus* an activity of 8–12 units/mg of dried venom (Fig. 3). The reverse is observed in the assay of the toxicity by measuring the survival times of injected mice. The venom from the *T. serrulatus* is two or three times more toxic than the *T. bahiensis* venom.

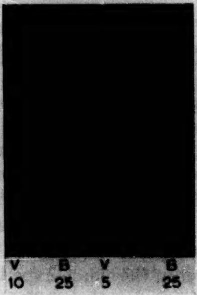

Fig. 3. Guinea pig ileum perfused with Tyrode solution in a 10-ml bath. Comparison of the effect of addition of the venom from *T. bahiensis* (V) with the effect produced by bradykinin (B). Lower figures are the doses (μg) of substances added into the bath. 1 mg of bradykinin preparation was equivalent to 5.2 units.

Zone electrophoresis separates the observed muscle effect of these venoms in two peaks of activity with different mobilities. The first component active on smooth muscle migrates in the paper 1.0–2.0 cm towards the cathode (9 V/cm, 10 h, pH 7.4, $\mu = 0.05$) and 10–13 cm in the starch (10 V/cm, 20 h, pH 7.4, $\mu = 0.05$). In the guinea pig ileum preparation the eluates of this component elicit, after a period of latency, a slow contraction of the longitudinal and circular layers of the intestine muscle, often followed by alternate periods of relaxation. Most of the toxic activity of the venoms is associated with this component. The eluates, when they are injected intraperitoneally into mice, produce the same symptoms of intoxication observed when the whole venom is injected, *i.e.*, excitation, lacrimation, salivation, sweating, dyspnoea and death. In general, the pharmacological effects of this component exhibited in mice and smooth muscle preparations mimic a parasympathetic stimulation and are similar to those already described[6] for a partially purified extract of the telsons from *T. serrulatus*. Because this component is so toxic it will be referred to below as Scorpiotoxin T (T for *Tityus*).

The second peak of smooth muscle activity can be observed in the eluates from paper segments cut 3–4 cm from the point of origin in the direction of the cathode. In the starch it migrates 17–18 cm from the point of origin in the same direction. The eluates elicit in the guinea pig ileum preparation, after a period of latency, a slow, persistent contraction resistant to several washings of the preparation. The type of the contraction is very similar to the contraction produced by bradykinin in this organ (Fig. 4), but it is somewhat slower. This component seems to be of low toxicity.

Fig. 4. Guinea pig ileum. Assay of 0.2 ml of solution of scorpiotoxin and scorpiokinin after 30 min incubation with boiled and unboiled trypsin. V = 7.5 μg of whole *T. bahiensis* venom. T and K = 0.2 ml of solutions of toxin and kinin incubated with boiled trypsin. T + T and K + T = 0.2 ml of solutions of toxin and kinin incubated with unboiled trypsin. The smooth muscle activities are destroyed by the proteolytic enzymes. The same result is observed with eluates obtained from electrophoresis of *T. serrulatus* venom.

Lyophilized eluates of this component, diluted to contain an amount equivalent to 200 μg of the total venom (assayed in the guinea pig ileum preparations) when injected in mice, do not cause any visible sign of intoxication excepting a short period of adynamia. This component will be referred to below as Scorpiokinin.

The recoveries of the toxic and smooth-muscle activities are low in the filter paper experiments, but are higher in the starch experiments. In a typical experiment 5.0 mg of the *T. bahiensis* venom were submitted to electrophoresis during a period of 20 h, starch paste being used as support; 82 % of the total smooth muscle activity was recovered in the eluates from segments of numbers 9 to 20, 53 % being contained in the scorptiotoxin, and 29 % in scorpiokinin, peaks. The toxic activity, tested by the mouse survival time, recovered in the scorpiotoxin peak accounted for 48 % of the initial total toxic value (Fig. 5).

Scorpiotoxin and scorpiokinin are probably of a polypeptide nature, because the toxic and smooth muscle activities of the whole venom are destroyed by chymotrypsin and trypsin. Inactivation was also demonstrated by using components separated by electrophoresis. Lyophilized starch eluates containing scorpiotoxin or scorpiokinin were dissolved in phosphate buffer, pH 7.4, assayed in the guinea pig ileum preparations, the whole venom being used as a standard of reference, and diluted to obtain a concentration equivalent to approx. 1 mg of whole venom in 5 ml of the solution. This solution was incubated with 0.1 mg of crystalline trypsin or chymotrypsin and

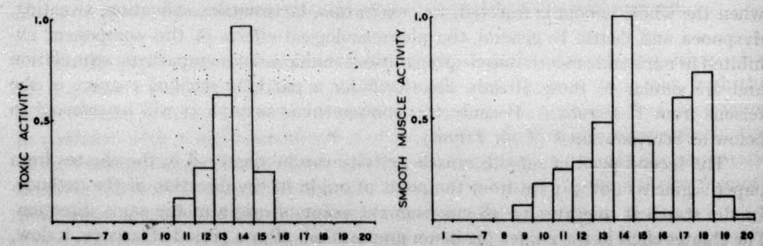

Fig. 5. Fractionation of 5.0 mg of *T. bahiensis* venom by starch electrophoresis. The number of 1 cm starch block is indicated in abscissae. Ordinates represent the toxic and muscle activities expressed in arbitrary units. 1 unit is the activity equivalent to 1 mg of whole venom used as standard of reference. Note the two peaks of smooth muscle activity and the single peak of toxicity.

assayed, at various intervals of time, for toxicity in mice and for smooth muscle activities in the guinea pig gut. Appropriate blanks were run with boiled enzymes and assayed in the same way. The results, shown in Fig. 6 and Table I, indicated a marked inactivation of scorpiotoxin and scorpiokinin by the pancreatic enzymes.

TABLE I

RECIPROCAL OF SURVIVAL TIME OF MICE INJECTED WITH SCORPIOTOXIN SOLUTIONS AFTER 30 min INCUBATION WITH 10 μg OF BOILED AND UNBOILED TRYPSIN AND CHYMOTRYPSIN

Equivalent of total venom in 0.2 ml solution	*Survival time in reciprocal minutes* $\times 10^4$*			
	Boiled trypsin	*Trypsin*	*Boiled chymotrypsin*	*Chymotrypsin*
50 μg	182	0	166	41
100 μg	384	16	353	160

* Results are the average of survival time from 4 injected mice (18–20 g). Toxin was obtained by elution from starch electrophoresis experiments.

It is worth noting that the *T. serrulatus* venom previously mentioned, which presented in paper electrophoresis the unusual protein pattern, showed only the activity on the smooth muscle associated with the toxic component (scorpiotoxin).

Permeability factor

The venom from *T. serrulatus* and *T. bahiensis* are active on the capillary vessels of the rat skin. 100 μg of the whole venom injected into the abdominal skin of the rat increases the permeability of the capillary vessels to Evans Blue dye previously administered to the animal. The dye extravasates 3–5 min after the injection of the venom, producing a blue ring around the injection site. The hind paw of the rat is more sensitive to this factor: the injection of 5.0 μg of the venom produces in a few minutes an intense oedema of the wheal with extravasation of the dye. The injection of the venom into the paw is painful. The rats cry out after the injection of the venom and withdraw their legs in a characteristic way, avoiding any contact with the surface of the cage.

Histamine is probably involved in this process. Phenergan (4 mg/kg rat weight) administered intravenously 30 min before the venom, inhibits the oedema and the extravasation of the dye. Dibenziline, (8 mg/kg weight), a sympatholytic drug, is also effective in this respect. We have observed, however, that this drug also antagonizes the effects of histamine on the capillary permeability.

Assay of paper eluates intradermally in rats showed that the permeability factor has a positive charge at a pH 7.4 and can also be separated as a protein component by this method (Fig. 2).

Enzymic activities

We have tested the following enzymic activities in the *Tityus* venoms: proteolytic, fibrinogen clotting, phospholipase (egg yolk coagulation test), hemolytic, hyaluronic acid depolymerization, acetylcholinesterase and acetylcholinesterase inhibition. Among the enzymes studied the only one showing considerable activity is hyaluronidase*. *T. serrulatus* has an activity of 2.3 V.R. units/mg and *T. bahiensis*, showed 11.0 V.R. units/mg. The localization of hyaluronidase activity in paper electrophorograms is indicated in the Fig. 2.

DISCUSSION

The results presented in this paper indicate that in the venom from the scorpions *T. serrulatus* and *T. bahiensis* there is a plurality of biological active components. A fraction, separated by zone electrophoresis, exceeds, however, the others in toxicity. Approx. 50 % of the toxic activity of these venoms can be recovered in this fraction (Scorpiotoxin). The remaining components are of low toxicity, but they can, in some way, enhance the toxicity of the main toxic component. The permeability factor and the hyaluronidase possibly help to increase the toxicity of the *Tityus* venoms by facilitating diffusion and absorption of the toxin by the lymphatic and blood vessels. In addition, histamine released by the permeability factor may contribute to the general effects of the poisoning in species known to be sensitive to this substance, such as the guinea pig and man.

The sting of *T. serrulatus* is responsible for most of the fatalities caused by scorpions in Brazil[13]. The results presented in this paper and the determination of the LD_{50} done by BÜCHERL[2] have shown that this venom is 2–3 times more toxic than the venom of *T. bahiensis*. The electrophoresis experiments have shown that both venoms are qualitatively very similar in regard to their protein composition and their content of biologically-active components. The difference in toxicity is probably due to a variation in the proportions of the various active components.

It is possible to separate, from the venoms of both species, a fraction which has a low toxicity, but is very active on the smooth muscle of the guinea pig ileum preparation. The name proposed for this substance, scorpiokinin, is used in the sense suggested recently by ROCHA E SILVA[14]. This author includes under the name of kinin all substances of a polypeptide nature which cause slow contraction of the smooth muscle of the guinea pig ileum. Scorpiokinin differs from bradykinin in that, unlike bradykinin, it is easily inactivated by trypsin and it is a weak stimulant of the smooth muscle of the rat uterus.

The question arises whether scorpiotoxin cannot also be considered to be a kinin, because its lethal component is associated with contractile activity and is also of a polypeptide nature. So far, we have not been able to dissociate these two activities in the toxic component, but an association of them cannot be excluded. This question is being investigated in our laboratory.

The sting of the scorpion is very painful and it will be of interest to investigate which fraction in the venom of these arachnids is responsible for the pain inflicted. There is, in wasp venom, in addition to histamine and serotonin, a substance with the properties of bradykinin which is, it is believed, responsible for the pain inflicted by this sting[15,16]. FISCHER AND BOHN[17], who have done electrophoretic studies of *Tityus* venoms and have analyzed total hydrolysates of scorpion venom, have not found histamine in these venoms. The considerable amount of serotonin found[18] in the venom of the African scorpion *Leiurus quinquestriatus* could not be detected in acetone extracts of *Tityus* venoms, either by a pharmacological test on rat uterus or in filter paper chromatograms.

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* We are indebted to Dr. REINALDO DE MEDEIROS MELO for hyaluronidase activity determinations.

The difference between the electrophoretic patterns observed in the two batches of venom from *T. serrulatus* cannot be easily explained. This difference is not seasonal because the venom of the same batch of scorpions gives the same electrophoretic pattern all the year round. Piza[19] has demonstrated chromosomic aberrations in *T. bahiensis* and some physiological alterations are to be expected in this species. This has not yet been observed in *T. serrulatus*.

There is, however, the possibility that slight differences in the methods of collecting the venoms or in the conditions of drying and storing them may alter the composition and the electrophoretic properties of the venoms. When anti-scorpionic serum is prepared, the existence of venoms with different antigenic compositions should be kept in mind, because variations of protein composition can be observed in venoms from the same species of scorpions.

ACKNOWLEDGEMENT

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THE CHLOROPHYLLS OF GREEN BACTERIA

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SUMMARY

Contrary to previous assumptions, at least two different molecular species of chlorophyll exist among the green bacteria. Two strains of *Chlorobium thiosulfatophilum* each contain a single, distinct type of chlorophyll. The spectral properties of these two pigments and of the corresponding pheophytins are described, together with certain of their chemical properties.

INTRODUCTION

It has long been recognized that the two groups of photosynthetic bacteria contain chlorophyllous pigments which differ from the chlorophylls of algae and higher plants. In all purple bacteria so far examined, there is a single molecular species of chlorophyll[1], which was identified over 20 years ago by FISCHER and his co-workers[2] as 2-desvinyl, 2-acetyl, dihydrochlorophyll *a*, and has been well characterized physically[3]. Observations on crude extracts from green bacteria have indicated that they contain another kind of chlorophyll, formerly termed bacterioviridin[4], and more recently designated by the name chlorobium chlorophyll[5]. The first absorption spectrum of carotenoid-free material was published only in 1955 by GOODWIN[6], who prepared this pigment from a strain of *Chlorobium thiosulfatophilum* isolated at Oxford by LASCELLES. GOODWIN described it as spectroscopically very similar to chlorophyll *a*, with main peaks in ether at 659, 431 and 408 mμ.

Some months ago, with the aim of characterizing chlorobium chlorophyll more precisely, we undertook its isolation from dried cells of *C. thiosulfatophilum*, kindly prepared for us at the Norges Tekniske Høgskole in Trondheim by LARSEN. Much to our surprise, the principal chlorophyllous pigment in these cells had an absorption spectrum markedly different from that reported by GOODWIN, showing main peaks in ether at 650, 612, 575, 425 and 406 mμ. Our observations were confirmed in LARSEN's laboratory on extracts from fresh cells, thus obviating the possibility that the pigment in question was a decomposition product formed from the native chlorophyll during the drying process. About this time, KAPLAN AND SILVERMAN[7] published a description of the spectral properties of the same pigment, which they had isolated from a culture of *C. thiosulfatophilum* sent to them by Larsen. Curiously enough, they made no comment on the marked discrepancies between their data and the earlier data of Goodwin.

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* Professor of the Miller Institute for Basic Research in Science of the University of California, 1957–1959.

The strain of *C. thiosulfatophilum* (strain L) maintained at Trondheim had been obtained from Lascelles, and was presumed by Larsen to be identical with that earlier furnished to Goodwin. Upon enquiry, however, Dr. Lascelles informed us that she had in fact isolated two strains, L and PM. Both conformed to Larsen's characterization of *C. thiosulfatophilum*[5], and no record had been kept of the particular strain furnished to Goodwin. Fortunately, both strains were still in existence at the time of our enquiry. Lascelles proceeded to determine the absorption spectra of the intact cells, and immediately observed a striking difference. In strain L the main peaks *in vivo* lie at 450 and 725 mμ; in strain PM, at 460 and 747 mμ (Fig. 1).

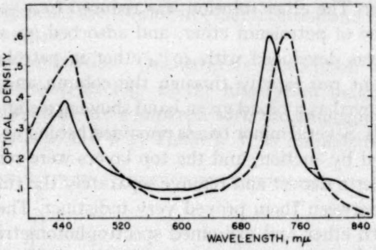

Fig. 1. Absorption spectra of whole cells of two strains of *Chlorobium thiosulfatophilum* (data of Dr. J. Lascelles). ———, strain L; – – – –, strain PM.

It has been known for many years that the position of the far red peak in the absorption spectrum of the cells of different strains of green bacteria is subject to some variation[5]; but this had been attributed to differences in the intracellular coupling of a single type of chlorophyll, corresponding to the situation among the various species of purple bacteria, where the position of the main infra-red peak of bacteriochlorophyll in whole cells and in chromatophores can vary by as much as 40 mμ. However, the marked discrepancies between the data of Goodwin and of later investigators on the spectral properties of extracted chlorobium chlorophyll suggested that the spectral differences between Lascelles' two strains of *C. thiosulfatophilum* might be caused by the presence of different kinds of chlorophyll. An examination of the chlorophyll extracted from dried cells of strain PM, kindly grown for us by Lascelles, immediately confirmed this supposition: the chlorophyll of strain PM had the spectral properties attributed to chlorobium chlorophyll by Goodwin, who must therefore have worked with this strain.

It thus became evident that the two strains of *Chlorobium thiosulfatophilum* isolated by Lascelles each contain a single, chemically different type of chlorophyll. We shall designate these pigments as chlorobium chlorophyll-650 and chlorobium chlorophyll-660, the numerical suffixes referring to the position of the red absorption maximum of the isolated pigment in ether. The isolation and some of the properties of these two pigments are described below.

Isolation of chlorobium chlorophylls

Isolation of chlorobium chlorophyll-650: 5 g of dried cells of *C. thiosulfatophilum* strain L were suspended in 20 ml of water and treated for 10 min in a 9 kc Raytheon sonic oscillator in order to disperse the material. The suspension was mixed with 4 volumes of methanol and centrifuged, the sediment being further extracted with successive portions of 80 % methanol until no further pigment was removed. An equal volume of ether was added to the combined methanol extracts in a separatory funnel, and the pigments were transferred to ether by the cautions addition of water. The ether layer was washed several times with water, and dried over Na_2SO_4. The ether solution was then reduced by evaporation to a volume of 20 ml, and mixed with 80 ml of petroleum ether. The bulk of the chlorophyll precipitated, and was collected on diatomaceous earth by suction, thoroughly washed with petroleum ether, and dissolved in ether. The ether solution was reduced to a volume of 50 ml, mixed with an equal volume of petroleum ether, and adsorbed on a column of powdered sugar. The column was developed with 40 % ether in petroleum ether. A trace of carotenoid still present ran rapidly through the column and was followed by the chlorophyll, which moved as a broad green band showing a slight apparent separation into three lesser bands. Several minor bands remained behind at the top of the column. The column was dried by suction, and the top layers were removed and discarded. An attempt was made to dissect and remove separately the three main green bands, but the boundaries between them proved very indistinct. The three fractions were separately eluted with ether and examined spectrophotometrically. Since the peak positions and relative peak heights were identical in all three, the ether solutions were pooled and set aside. A second batch of pigment was prepared in the same way, after which the two preparations were combined and rechromatographed in the same fashion. Development of this column with 40 % ether in petroleum ether showed a single broad green band, which separated well from minor impurities (decomposition products?) that were held at the top of the column. The column was dried, and the main green band was eluted with ether.

The pheophytin of this pigment was prepared by the procedure described by Smith and Benitez[3] for the preparation of bacteriopheophytin from bacteriochlorophyll. It was subsequently purified by adsorption on sugar and development with 40 % ether in petroleum ether.

Isolation of chlorobium chlorophyll-660: 5 g of dried cells of *C. thiosulfatophilum* strain PM were ground in a mortar with about 20 ml of 80 % methanol. The extract was filtered, and the residue was repeatedly extracted with additional portions of 80 % methanol until no further pigment was removed. The combined filtrates were mixed with ether in a separatory funnel, and the pigments were transferred to the ether layer by the addition of water. The ether solution was washed with water and dried over Na_2SO_4. It was then evaporated to a volume of 18 ml, mixed with 3 volumes of petroleum ether to precipitate the chlorophyll, and filtered over talc. The precipitated pigment was well washed with petroleum ether and redissolved in ether. This solution was evaporated to a volume of 55 ml, mixed with 100 ml of petroleum ether, and adsorbed on a sugar column, which was developed with 50 % ether in petroleum ether. Development was continued until a narrow purple band had passed through the column. The column was then dried, the top layers were removed and discarded, and the very broad main green band was eluted with ether. After volume reduction, this ether solution was mixed with an equal volume of petroleum ether, again adsorbed on sugar, and developed first with 50 % ether in petroleum ether and then with 30 % ether in petroleum ether. Several minor purple and green bands passed through the column. The column was then dried, and the main green band was carefully dissected out, redissolved in ether and chromatographed a third time on sugar.

The purple pigment which had passed through the first sugar column consisted mainly of the pheophytin of chlorobium chlorophyll-660 (see ref. 6). This solution was rechromatographed on sugar, the column being developed with 30 % ether in petroleum ether. The principal, dark grey leading band was dissected out and eluted with ether.

Physical and chemical properties of the chlorobium chlorophylls

Solubility: Both pigments are soluble in ether, acetone and methanol; completely insoluble in petroleum ether.

Spectral absorption properties: Quantitative absorption curves for the two pigments in ether are shown in Fig. 2; the absorption maxima and the specific absorption coefficients in ether are compared with the analogous data of SMITH AND BENITEZ[3] for chlorophylls *a* and *b* in Table I. It is immediately evident that the

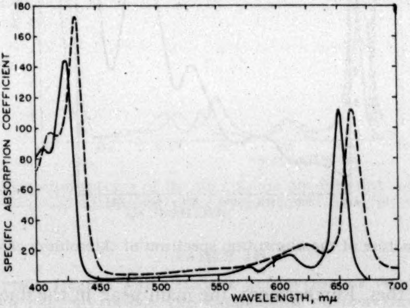

Fig. 2. Quantitive absorption spectra of the two chlorobium chlorophylls in ether. ———, chlorobium chlorophyll-650. – – – – –, chlorobium chlorophyll-660.

TABLE I

ABSORPTION MAXIMA AND SPECIFIC ABSORPTION COEFFICIENTS OF THE CHLOROBIUM CHLOROPHYLLS, COMPARED WITH THOSE OF CHLOROPHYLLS *a* AND *b*

Solvent: Ether

Pigment	*I*	*II*	*III*	*IV*	*V*	*VI*	
Chlorophyll *a*	662	615	578	533.5	430	410	mμ
	101	16.3	9.3	4.2	131.5	85.2	l./g cm
Chlorobium-chlorophyll-650	650	612	575	530	425	406	mμ
	113.5	15.8	8.6	3.9	146.0	87.2	l./g cm
Chlorobium-chlorophyll-660	660	622	—	—	432	412	mμ
	112.5	19.7	—	—	175.5	89.7	l./g cm
Chlorophyll *b*	644	595	549	—	455	430	mμ
	62.0	12.7	7.1	—	174.8	62.7	l./g cm

chlorobium chlorophylls resemble chlorophyll *a* in spectral structure, in relative peak height and in absolute light absorption at the major peaks. The spectral differences among the three pigments reside primarily in the exact positions of the maxima, and in the degree of resolution of the series of minor peaks that lie between the Soret peak and the red peak. Chlorobium chlorophyll-650 shows the sharpest spectral structure in this region (Fig. 3), with a double peak between 600 and 615 mμ; only a single peak occurs in the comparable region of the spectra of chlorophyll *a* and chlorobium chlorophyll-660. It can be seen from Fig. 2 that light absorption by our preparation of chlorobium chlorophyll-660 does not drop to zero in the far red region. In fact, it rises again beyond 700 mμ to a very broad, low maximum at about 750 mμ. A similar observation was reported by GOODWIN[5], who gave a position of 765 mμ for the maximum. We have observed that the height of this peak increases, with a concomitant drop in the heights of the major peaks, when a solution of the pigment is allowed to stand in ether. Furthermore, the main peak in the fluorescence spectrum lies at 663 mμ, and thus clearly corresponds to the main red peak in the absorption spectrum at 660 mμ. For these reasons, we infer that the small infra-red peak does not belong to the absorption spectrum of chlorobium chlorophyll-660 itself, but reflects the presence of a decomposition product, formed in small amounts during the isolation of the chlorophyll. Chlorobium chlorophyll-660 is exceptionally sensitive to destruction by light, and since we were unaware of this fact until after its isolation had been completed, the exposure received during the manipulations of chromatography may well have been sufficient to cause slight decomposition.

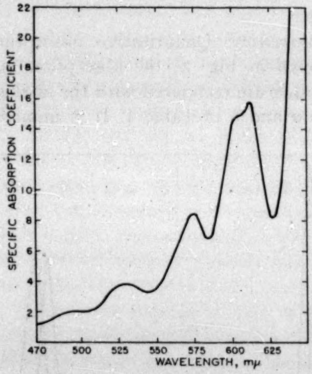

Fig. 3. Fine structure of the absorption spectrum of chlorobium chlorophyll-650.

Table II presents the absorption maxima and specific absorption coefficients of the two chlorobium chlorophylls in methanol and acetone.

Fluorescence maxima: The main fluorescence maximum of chlorobium chlorophyll-660 in ether solution lies at 663 mμ; that of chlorobium chlorophyll-650 at 653 mμ.

Magnesium content: The magnesium contents of the purified pigments were determined by the Titan Yellow method[3]. On a dry weight basis, chlorobium chloro-

TABLE II

ABSORPTION MAXIMA AND SPECIFIC ABSORPTION COEFFICIENTS OF THE CHLOROBIUM CHLOROPHYLLS IN METHANOL AND ACETONE

Pigment	*Solvent*	*I*	*II*	*III*	*IV*	*V*	*VI*	
Chlorobium chlorophyll-650	Methanol	659	612.5	—	—	427	411	mμ
		82.3	18.0	—	—	83.5	76.5	l./g cm
	Acetone	654	612.5	577.5	530	427	406	mμ
		98.0	16.4	9.2	3.9	125.9	87.9	l./g cm
Chlorobium chlorophyll-660	Methanol	670	620	—	—	435	419	mμ
		86.0	18.1	—	—	97.8	86.1	l./g cm
	Acetone	662.5	625	—	—	433	413	mμ
		92.6	16.9	—	—	142.8	88.8	l./g cm

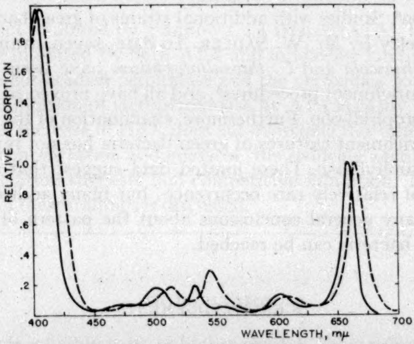

Fig. 4. Qualitative absorption spectra of the chlorobium pheophytins, adjusted to the same O.D. at the Soret peaks. ———, chlorobium pheophytin-650; – – – – –, chlorobium pheophytin-660.

TABLE III

ABSORPTION MAXIMA AND RELATIVE ABSORPTION OF PHEOPHYTIN *a*, CHLOROBIUM PHEOPHYTIN-650 AND CHLOROBIUM PHEOPHYTIN-660 IN ETHER

Pigment	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	
Pheophytin *a*	667	609.5	560	534	505	471	408.5	mμ
	48.4	7.4	2.7	9.5	11.0	3.9	100	rel. abs.
Pheophytin-650	660	605	555	532	503	470	405	mμ
	49.4	6.6	2.5	9.8	8.9	3.1	100	rel. abs.
Pheophytin 660	665	607	—	545	514	482	408	mμ
	47.9	5.7	—	13.3	8.9	3.3	100	rel. abs.

phyll-660 contained 2.92% magnesium, and chlorobium chlorophyll-650, 2.59%.

Phase test: This was performed as described by Smith and Benitez[3]. Despite repeated attempts, a positive test was never obtained with either pigment.

Absorption spectra of the pheophytins: Qualitative absorption spectra of the pheophytins of the two chlorobium chlorophylls are shown in Fig. 4; for purposes of comparison, they were adjusted to the same absorbances at the Soret peaks. Absorption maxima and relative absorbances for these two pigments in ether are compared with the values for pheophytin *a*[3] in Table III; again, the broad similarities among the three pigments are strikingly evident.

DISCUSSION

The assumption, heretofore generally made, that all green bacteria contain a single molecular species of chlorophyll, is not correct. Two strains of green bacteria which both belong on present taxonomic criteria to the species *Chlorobium thiosulfatophilum* contain chemically different chlorophyllous pigments, a single molecular type occurring in each strain. This is a situation without parallel in any other major group of photosynthetic organisms; as a general rule, the chlorophylls present in the members of a major group are so constant as to provide a characteristic chemical hallmark of the group in question[8]. Studies with additional strains of green bacteria are now being carried out in Berkeley by Mr. W. SADLER. To date, seven strains belonging to the species *Chlorobium limicola* and *C. thiosulfatophilum* have been isolated from local muds by standard enrichment procedures[5], and all have proved to contain exclusively the chlorobium chlorophyll-660. Furthermore, examination of the pigment in a series of six additional enrichment cultures of green bacteria has not revealed the presence of chlorobium chlorophyll-650. These limited data suggest that chlorobium chlorophyll-650 may be of relatively rare occurrence; but many additional isolations will be required before any general conclusions about the pattern of chlorophyll distribution in the green bacteria can be reached.

ACKNOWLEDGEMENTS

The authors are deeply indebted to Professor H. LARSEN and Dr. J. LASCELLES for their help in the provision of biological material, and in the performance of observations essential for the elucidation of this problem. They also wish to thank Dr. S. FRENCH for determining the fluorescence spectra.

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INHIBITION OF SOME METALLOPROTEIN ENZYMES BY 3-AMINO-1,2,4-TRIAZOLE

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SUMMARY

Glucose oxidase is not inhibited by 3-amino-1,2,4-triazole(ATA). Tyrosinase and fatty acid peroxidase are inhibited by both ATA and imidazole. Lactoperoxidase is inhibited by ATA while horseradish peroxidase and the peroxidase found in seedlings are not inhibited. The inhibition of lactoperoxidase by ATA is easily reversed by dialysis against dilute saline. Increase in H_2O_2 does not overcome this inhibition. The relationship between ATA concentration and guaiacol (the reducing agent used in the peroxidase assay) concentration suggests non-competitive inhibition. The possible physiological significance of these findings is discussed.

INTRODUCTION

3-amino 1,2,4-triazole is highly toxic to higher plants[1]. The syndrome of ATA toxicity consists of growth inhibition, low chlorophyll level and few and misshapen chloroplasts[2]. In higher animals prolonged intake of ATA causes enlargement of the thyroid[3]. The influence of ATA upon several enzyme systems has been studied both *in vivo* and *in vitro*. Plants treated with ATA showed subnormal catalase activity[4,5]. Nutlets of Nut Grass (*Cyperus rotundus* L.) germinated in the presence of ATA had a lower level of ascorbic acid oxidase, DOPA oxidase, peroxidase and catalase activities than the controls[6]. In rats treated with ATA liver and kidney catalase were markedly depressed, and liver peroxidase slightly depressed, while blood catalase, hemoglobin, and liver cytochrome *c* remained normal[7].

MARGOLIASH AND NOVOGRODSKY[8] have studied the inactivation of purified ox liver catalase and human erythrocyte catalase and have concluded that ATA reacts with complex I between catalase and hydrogen peroxide, leading to a complete inhibition of the catalatic activity[8,9]. The reaction was slow, was prevented by ferricyanide, and the catalase thus inactivated could not be reactivated by prolonged dialysis. PAUL AND MARGOLIASH[9] found that ATA has no inhibitory effect upon horseradish peroxidase, but that the latter catalyses a slow oxidation of ATA to unidentified products.

This report describes studies on the effect of ATA upon fatty acid peroxidase,

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Abbreviations used: ATA, 3-amino-1,2,4-triazole; DOPA, dihydroxyphenylalanine; Tris, (tris)hydroxymethylaminomethane; butyryl-AMP, butyryl adenylate.

glucose oxidase, tyrosinase, lactoperoxidase and peroxidases from various plant sources.

MATERIALS

The following commercially available enzymes were used: L-amino acid oxidase, *Crotalus adamanteus* venum, Ross Allen's Reptile Institute, Silver Springs, Fla.*, Glucose oxidase Worthington Biochemical Corporation, Freehold, N. J.*, Tyrosinase, Sigma Chemical Co., 3500 DeKalb St., St. Louis 18, Mo.*, Horseradish peroxidase, Sigma Chemical Co., 3500 DeKalb St., St. Louis 18, Mo.*.

Whey used as a source of lactoperoxidase was prepared by treating 250 ml of raw skim milk** with 1 ml of commercial rennin** for 2 h at 30°. The curd was removed by straining through cheese cloth. The whey was kept frozen when not in use. No loss in activity was observed over a month.

Fatty acid peroxidase: Peanuts (*Arachis hypogea* L. var. Virginia Jumbo*) were germinated for 7 days in the dark in vermiculite. The cotyledons were blended for 30 sec with 2 volumes of ice-cold 0.2 *M* Tris buffer pH 7.35. The brei was strained through cheese cloth and the filtrate was centrifuged for 30 min at 30,000 × *g*, at 0°, in the Servall Refrigerated Centrifuge.

Corn, Sunflower and Kidney Bean peroxidases: Corn (*Zea mays*), sunflower (*Helianthus annuus* L.) and kidney bean (*Phaseolus vulgaris*) were germinated in vermiculite in the dark for 5, 5, and 7 days, respectively. The seed and root were removed and the stem portion (including the coleoptile in the case of corn) was ground with two volumes of 0.1 *M* sodium phosphate buffer pH 6.8 in a pre-chilled mortar containing sand. The brei was centrifuged 30 min at 30,000 × *g*, 0°, in the Servall Refrigerated Centrifuge.

The supernatants contained peroxidase activity, which was stable over several weeks. The preparations were kept frozen when not in use.

ATA, American Cyanamid, Technical, was recrystallized twice from abs. Ethanol m.p. 152°. Imidazole, Eastman. Ammonium [1-^{14}C]palmitate was prepared from [1-^{14}C] palmitic acid, Tracerlab[10]. L-leucine: Fisher Scientific Co., Fisher reagent grade. Guaiacol: Eastman. D-glucose: Eastman. Catechol: Nutritional Biochemical Co. Ascorbic Acid: Eastman, Benzene: Baker Chemical Co., Baker Analyzed Grade. Potassium ferricyanide: Baker Chemical Co., Trishydroxymethylaminomethane Sigma 121: Sigma Chemical Co.

Buffers: 0.01 *M* sodium phosphate buffer pH 6.8; 0.2 *M* Tris hydrochloride buffer, pH 7.35; 0.1 *M* Tris, 0.1 *M* KCl buffer pH 7.2; 0.4 *M* Na_2HPO_4–0.2 *M* citric acid buffer pH 5.1.

METHODS

Fatty acid peroxidase was assayed by counting the CO_2 liberated from [1-^{14}C] palmitate as previously described[11]. Two H_2O_2 generating systems were used in this assay, 2 mg of glucose oxidase plus 10 μmoles of D-glucose/2 ml of total volume, or 1 mg of L-amino acid oxidase plus 10 μmoles of L-leucine/2 ml of total volume[12].

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* Gracious gift of the Department of Biochemistry, University of California Davis, Davis, Calif. (U.S.A.).

** Gracious gift of the Department of Food Science and Technology, University of California Davis, Davis, Calif. (U.S.A.).

Glucose oxidase was assayed manometrically using a standard Warburg respirometer. Tyrosinase Sigma was assayed for catecholase activity by the method of DAWSON AND MAGEE, the time required for the oxidation of 1.71 µmoles of ascorbic acid being determined in each case[13].

Peroxidase activity was determined as follows: 20 µmoles of guaiacol, 10 µmoles of D-glucose, 2 mg of glucose oxidase and other substances under study were mixed in a 30-ml beaker; enough 0.01 *M* sodium phosphate buffer, pH 6.8 was added to bring the total contents to 2.0 ml. The reaction was started by the addition of peroxidase and the beaker was placed in the dark. After exactly 5 min the solution was poured into a 12-ml glass centrifuge tube contained in a metal cup, and 5 ml of benzene was added from an automatic pipette. The tube was covered with aluminium foil to screen off the light, shaken by hand, and centrifuged at moderate speed to break up the emulsion. The benzene layer was transferred by means of a medicine eyedropper to a corax cell and the color was read at 470 mµ in the Beckman D.U. Spectrophotometer. The instrument was balanced against a suitable reagent blank. The color is stable for several hours in the dark but fades rapidly if exposed to light.

RESULTS

ATA was found to inhibit fatty acid peroxidase as shown in Table I. Imidazole and 1,2,4-triazole are known to be effective inhibitors of this enzyme, while other substituted analogues have little, if any, effect[14]. The hydrogen peroxide generating system, D-glucose-glucose oxidase is not affected by ATA as shown in Fig. 1.

TABLE I

INHIBITION OF FATTY ACID PEROXIDASE BY ATA AND IMIDAZOLE

Each vessel contained: In the main compartment, 10 µmoles of D-glucose and 2 mg of glucose oxidase or 10 µmoles of L-leucine and 1 mg of L-amino acid oxidase, 0.1 µmole of ammonium [1-^{14}C]palmitate, 0.5 ml of fatty acid peroxidase, ATA or imidazole and sufficient Tris–KCl buffer, to bring the total contents to 2.0 ml. In the center well, 0.20 ml of 20 % KOH. In the side arm, 0.35 ml 6 *N* H_2SO_4. The vessels were shaken for 90 min at 28°.

H_2O_2 generating system	*ATA* $M \times 10^4$	*Imidazole* $M \times 10^4$	*Counts/min in $BaCO_3$*	*Per cent ^{14}C in $BaCO_3$*	*Per cent inhibition*
D-glucose	—	—	3290	65.4	—
+ glucose oxidase	50	—	480	9.5	87
	—	50	340	6.8	91
	—	—	4223	83.8	—
L-leucine	50	—	672	13.4	84
+ L-amino acid oxidase	5	—	2823	56.1	33
	—	50	953	19.0	77
	—	5	2748	54.8	35

The effect of ATA and imidazole upon the catecholase activity of a purified tyrosinase preparation is shown in Table II.

Fig. 2 shows the relationship between the O.D. at 470 mµ and the concentration of lactoperoxidase in the assay.

In sharp contrast to horseradish peroxidase and to the peroxidases found in corn, sunflower and kidney bean seedlings, lactoperoxidase is inhibited by ATA. Imidazole

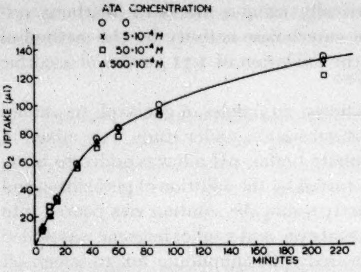


Fig. 1. Oxygen uptake by glucose oxidase–glucose in presence of ATA. Each vessel contained: In the main compartment, ATA as shown, 10 μmoles of D-glucose, Tris–KCl buffer to the total volume of 1.7 ml. In the side arm, 0.1 ml solution containing 2 mg of glucose oxidase, 0.2 ml of Tris–KCl buffer. In the center well, 0.2 ml of 20% KOH. After thermal equilibration, the reaction was started by tipping in the contents of the side arm. Temperature, 28°.

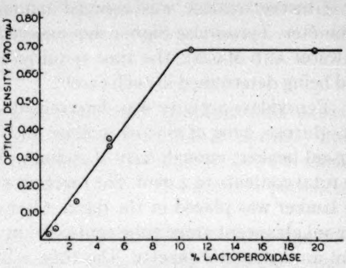


Fig. 2. Relation between O.D. and lactoperoxidase concentration.

TABLE II

INHIBITION OF CATECHOLASE ACTIVITY BY ATA AND IMIDAZOLE

Each vessel contained 1.0 ml of Na_2HPO_4–citric acid buffer, 0.05 mg of tyrosinase Sigma, 1.71 μmoles of ascorbic acid, 45.5 μmoles of catechol, H_2O to bring total to 10.0 ml. Reaction at room temperature in open 50-ml Erlenmeyer. Every 15 sec an aliquot of the reaction mixture was added to an equal volume of starch iodide indicator solution[13]. The end point is the appearance of a blue color in the test solution.

ATA $M \times 10^4$	*Imidazole* $M \times 10^4$	*µmoles ascorbate oxidized/min*	*Per cent inhibition*
—	—	0.684	—
500	—	0.190	72
50	—	0.428	37
	500	0.190	72
	50	0.380	44

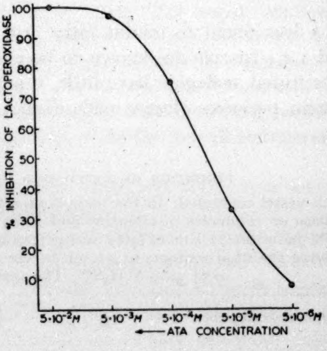


Fig. 3. Inhibition of lactoperoxidase by ATA.

has no effect on any of these enzymes. Fig. 3 represents graphically the inhibition of lactoperoxidase by ATA.

A lactoperoxidase preparation containing 20 μmoles of ATA/ml was dialyzed against 400 volumes of 0.85 % NaCl at 0°. At the same time a control without ATA was given the identical treatment. At 1-h intervals a 0.2-ml aliquot of each preparation was tested for peroxidase activity. No attempt was made to correct for volume changes during dialysis. The results are shown in Table III. It is clear that lactoperoxidase regains activity as soon as ATA is dialyzed.

The hydrogen peroxide generating system was enhanced by means of a tenfold increase in either D-glucose, glucose oxidase or both. These changes did not effect any reversal of ATA inhibition. The interaction between guaiacol and ATA in the assay for peroxidase activity has been studied. Fig. 4 summarizes the results of this study in graphical form.

TABLE III

REACTIVATION OF ATA-INHIBITED LACTOPEROXIDASE BY DIALYSIS AGAINST 0.85% NaCl

Time (h)	$(O.D.)_{470\ m\mu}$	
	ATA	*Control*
0	0.040	0.510
1	0.252	0.360
2	0.301	0.366
3	0.425	0.467

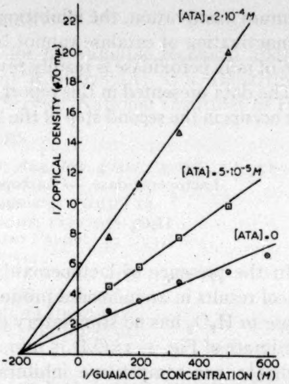


Fig. 4. Relationship between ATA and guaiacol concentration in the assay for lactoperoxidase.

Ferricyanide has been reported to prevent the inactivation of catalase by H_2O_2 and ATA[8]. This anion could not be tested in the lactoperoxidase–guaiacol system since it causes a rapid, non-enzymic oxidation of guaiacol. On the fatty acid peroxidase system, however, ferricyanide has no effect. No fatty acid peroxidase activity could be demonstrated in milk.

When *p*-phenylene diamine is substituted for guaiacol as the hydrogen donor, the oxidation catalysed by lactoperoxidase is still blocked by the addition of ATA. However, on the oxidation of *p*-phenylene diamine by H_2O_2, catalyzed by cupric ion, ATA has no inhibitory effect.

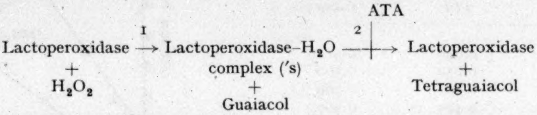
DISCUSSION

ATA and 1,2,4-triazole have no observable effect upon the rate of breakdown of acetyl adenylate, while under the same conditions imidazole causes a destruction of acetyl adenylate which is too rapid to measure[15]. No evidence exists that the fatty acid peroxidase involves an activation step. Indeed MARTIN AND STUMPF[14] found that imidazole does not inhibit the β-oxidation of butyric acid in peanut mitochondria, where the active intermediates butyryl-AMP and butyryl-S-CoA are undoubtedly involved, while it does inhibit the fatty acid peroxidase system in the same particles. Therefore it appears likely that some other mechanism than acyl group transfer is responsible for the inhibition of fatty acid peroxidase by imidazole, triazole and ATA. This mechanism may involve the formation of a complex between the iron porphyrin of the peroxidase and the basic nitrogen of the inhibitor molecule.

The inhibition of purified tyrosinase occurs at a relatively high level of ATA and imidazole, a concentration of the order of 0.01 *M* being required for 50% inhibition. In this connection should be mentioned that both compounds undergo a reaction with cupric chloride indicated by a change in color. Therefore complex formation with the metal prosthetic group could also be responsible for the inhibition of tyrosinase.

The inhibition of lactoperoxidase differs in several respects from the inactivation of catalase studied by MARGOLIASH AND NOVOGRODSKY[8]. While the inactivation of catalase is a slow process requiring about 2 h of exposure to ATA and H_2O_2 for maximum inactivation, the inhibition of lactoperoxidase appears to be instantaneous. The inactivation of catalase cannot be reversed by prolonged dialysis while the inhibition of milk peroxidase is readily reversed.

The data presented in this report are consistent with the hypothesis that the ATA block occurs in the second step of the following reaction scheme,



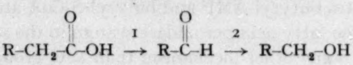
$$\begin{array}{ccccc} & & & \text{ATA} & \\ & 1 & & 2 & \\ \text{Lactoperoxidase} & \longrightarrow & \text{Lactoperoxidase–}H_2O & \nrightarrow & \text{Lactoperoxidase} \\ + & & \text{complex ('s)} & & + \\ H_2O_2 & & + & & \text{Tetraguaiacol} \\ & & \text{Guaiacol} & & \end{array}$$

In the presence of lactoperoxidase partially inhibited by ATA, an increase in guaiacol results in an increased production of the colored oxidation product, while an increase in H_2O_2 has no stimulatory effect. Assuming that the quantity plotted along the ordinate of Fig. 4, 1/(O.D.)5 min, is proportional to $1/V$, the data in Fig. 4 appear to indicate non-competitive inhibition[16]. Experiments are in progress with purified lactoperoxidase aimed to ascertain the specific mechanism of this inhibition.

The striking difference between the behavior of lactoperoxidase and that of horseradish and seedling peroxidases with respect to ATA probably hinges upon the differences in prosthetic groups. The prosthetic group of horseradish peroxidase (and most likely that of other common plant peroxidases) is protohematin IX (see ref. 17). The prosthetic group of lactoperoxidase is not known. On the basis of spectroscopic data Morell[18] suggested that it is a hematin closely related to protohematin but not identical with it.

The most striking physiological effects of ATA are: chlorosis and growth inhibition in plants[1], inhibition of production of thyroid hormone in mammals leading to thyroid enlargement[3], perhaps by a process of over-compensation. Recently Alexander[19] has shown that the incorporation of ^{131}I by homogenates of thyroid and salivary glands is dependent upon the presence of a H_2O_2 generating system, and is inhibited by ATA.

The inhibition of plant growth may be related to the effect of ATA on fatty acid peroxidase. Vlitos and Crosby[20] have shown that higher fatty alcohols are present in tobacco leaves and that they exert a growth-promoting influence comparable to that of indoleacetic acid. Higher fatty alcohols can be produced in plant tissue from fatty acids by means of the following two step synthetic pathway,



$$R\text{–}CH_2\text{–}\overset{\overset{O}{\|}}{C}\text{–}OH \xrightarrow{1} R\text{–}\overset{\overset{O}{\|}}{C}\text{–}H \xrightarrow{2} R\text{–}CH_2\text{–}OH$$

The first step is catalyzed by fatty acid peroxidase[14], the second step is catalyzed by a higher alcohol dehydrogenase which is widely distributed in plant tissue[21].

Research in this laboratory is now aiming to ascertain whether developing plant tissues contain any ATA-sensitive hydroperoxidases which play a role in the synthesis of chlorophyll and chloroplasts.

NOTE ADDED IN PROOF

Recently E. Margoliash *et al.*[22] have considered the reversible inhibition of catalase by ATA in connection with their study on the irreversible inactivation. From their data catalase appears to be approximately 500 times less susceptible of reversible inhibition by ATA than lactoperoxidase.

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STUDIES ON THE ELECTRON TRANSFER SYSTEM

XXIX. THE ISOLATION AND PROPERTIES OF A SUCCINIC DEHYDROGENASE–CYTOCHROME *b* COMPLEX FROM BEEF-HEART MITOCHONDRIA

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SUMMARY

1. The isolation and properties of a succinic dehydrogenase–cytochrome *b* particle from beef heart mitochondria are described.

2. The relation of cytochrome *b* to the main chain of terminal electron transport is discussed.

3. A method for assaying succinic-Coenzyme Q reductase activity is described.

Abbreviations: SDC, succinic dehydrogenase complex; $fs(b)_2$, succinic dehydrogenase–cytochrome *b*; CoQ, Coenzyme Q.

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INTRODUCTION

It has been demonstrated that cytochrome *b* can be preferentially reduced in an antimycin A-blocked mitochondrial system by succinate[1], an observation which suggests that cytochrome *b* and the succinic flavoprotein are closely allied in the terminal electron transport system. A partially purified cytochrome *b* preparation from beef heart[2] has been shown to retain considerable succinic dehydrogenase activity. The isolation of a functionally-intact succinic dehydrogenase–cytochrome *b* complex from *Propionibacterium pentosaceum*[3] lends further support to this hypothesis. The purpose of this communication is: (a) to describe the isolation and properties of an enzymically active succinic dehydrogenase–cytochrome *b* complex from beef heart mitochondria and (b) to demonstrate that this particulate complex catalyzes the rapid reduction of CoQ by succinate—a reaction in which cytochrome *b* is not an obligatory oxido-reduction component.

A preliminary report of this work has appeared earlier[4].

METHODS

Flavin, heme and non-heme iron were determined by methods previously described[5,6]. Protein was estimated by the biuret method[7]. Lipide content was determined by extracting the dried preparation (dried at 100°) with hot ethanol (50°). The ethanol extract was evaporated and its residue extracted with petroleum ether. After evaporation of the petroleum ether, the weight of the lipide was determined. The lipide values are expressed as the percent of the total dry weight (biuret protein plus lipide) which was extractable into hot ethanol and then into petroleum ether.

The Coenzyme Q_{10} content of the particle was measured by saponification and extraction into cyclohexane[8] as well as by the acid-phosphate method described by GREEN *et al.*[9].

Purified succinic dehydrogenase was prepared by the method of SINGER *et al.*[10], as modified by BASFORD *et al.*[11].

Succinic-ferricyanide, succinic-cytochrome *c* reductase and succinic-indophenol activities were measured as described previously[5].

Succinic-CoQ reductase activity was measured by the following method. An emulsion of CoQ_{10} in a solution of sucrose was prepared by adding 8.0 ml of 0.25 *M* sucrose to 1 mg CoQ dissolved in 0.2 ml of absolute ethanol. 4 µl of Triton X-100, 0.5 ml of 1.0 *M* potassium phosphate (pH 7.0), and 0.3 ml of 1.0 *M* succinate were added to the emulsion. The Triton is required to stabilize the emulsion, which is stable for several hours at room temperature with the detergent added. Aliquots (usually 0.47 ml) of the emulsion were transferred to test tubes and incubated for 5 min in a constant temperature bath at 38°. The reaction was started by adding 0.01 to 1.0 µg enzyme in 1 µl of 0.25 *M* sucrose. After 4 to 10 min the reaction was stopped by adding 0.1 ml of 1.0 *M* trichloracetic acid. In the control either the enzyme or substrate was added after the TCA. The $CoQH_2$ formed during the reaction was estimated spectrophotometrically by adding the following reagents in the order stated: 0.1 ml of 1.0 *M* sodium bicarbonate, 0.1 ml of 20% (w/v) Triton X-100, 0.14 ml of 3.0 *M* potassium phosphate buffer, pH 7.0, and 0.08 ml of 0.02% 2,6-dichlorophenol-indophenol. The bicarbonate and phosphate buffer neutralize the TCA and the detergent dissolves the precipitated protein. Reduced CoQ reacts quantitatively with indophenol and the reaction is complete in 10 min at room temperature or in 2–3 min at 38°. The solutions were transferred to 1-ml cuvettes and read at 600 mμ against a water blank in a Beckman DU spectrophotometer. The QH_2 produced during the enzymic reaction was calculated from the difference in O.D. of the control minus the experimental. The molar extinction coefficient ($18.7 \cdot 10^6$ $cm^{-1} \times mole^{-1}$) of indophenol under the condition of the assay was determined by titrating the dye with a standard QH_2 solution prepared by the method previously described[12]. The reduction of indophenol at pH 7.0 was a linear function of the $CoQH_2$ added until at least 98 % of the dye was reduced.

Reagents

The following reagents were obtained from the sources denoted in parentheses: Cytochrome *c* (Sigma Chemical Company); succinic acid and Coenzyme Q (Merck and Company); cholic acid and 2,6-dichlorophenol-indophenol (Eastman Chemical Company). Triton X-100 was a gift of the Rohm and Haas Company, Philadelphia, Pa.

Preparation of the particle

Preparation of the hemoprotein solution from the succinic dehydrogenase complex: The stage II SDC particle was prepared by methods previously described[6]. All manipulations were carried out at 0 to 5° unless otherwise indicated. The protein concentration of the particle suspension was adjusted to 20–30 mg/ml with sucrose–succinate solution (0.25 *M* sucrose, 0.005 *M* potassium succinate). 2 mg of potassium cholate/mg of protein were added and the solution was made 3 *M* with respect to KCl. The pH was maintained at 7.4. The solution was warmed slightly to permit all the KCl to go into solution and then centrifuged in the No. 30 rotor of a Model L Spinco preparative ultracentrifuge at 30,000 rev./min for 30 min. The precipitate was discarded and the clear, deep red supernatant was dialyzed for 5 h against 10 volumes of 0.01 *M* phosphate buffer, pH 7.4. The precipitate that developed during dialysis was removed by centrifugation. Solid ammonium sulfate, 10.6 g/100 ml, was added to the supernatant and the precipitate that formed was collected by centrifugation and discarded. An additional 20.7 g of solid ammonium sulfate/100 ml of solution was then added to bring the solution to 55 % saturation. The precipitate was collected by centrifugation and dissolved in 0.1 *M* phosphate buffer, pH 7.4. This fraction will be referred to as the hemoprotein solution. The fractionation to this point is essentially that reported by JÄRNEFELT *et al.*[13].

Extraction of the hemoprotein solution with petroleum ether: The clear hemoprotein solution was mixed with an equal volume of cold (0°) petroleum ether with vigorous stirring. It was important to keep the solutions as close to 0° as possible at this stage to insure fully active preparations. The opalescent, free flowing emulsion was centrifuged at 15,000 rev./min for 10 min in the preparative ultracentrifuge and the turbid aqueous layer collected with as little transfer of the organic layer as possible. The aqueous layer was then centrifuged in the No. 40 rotor of the preparative ultracentrifuge for 30 min at 40,000 rev./min. A translucent, deep red pellet formed which contained primarily succinic dehydrogenase and cytochrome *b* contaminated with variable amounts of cytochromes c_1 and *c*. The precipitate was washed at least twice with 10 volumes of neutralized 0.25 *M* sucrose. All or most of the cytochromes c_1 and *c* was removed in these washings. The particles were resuspended in 0.25 M sucrose and assayed for enzymic activity immediately. The particulate complex will hereafter be referred to as the fs(b)$_2$ particle.

RESULTS

Composition

The particulate preparations obtained by the methods described contained 3.2 to 3.4 mμmoles cytochrome *b*, 6.0 to 6.4 mμmoles non-heme iron, and 1.6 to 1.7 mμmoles of flavin/mg of protein (*cf.* Table I). Tryptic digestion was a necessary preliminary for the release of flavin. The preparations were free of acid extractable flavin and cytochrome *a*, but occasionally small amounts of cytochromes c_1 or c (0.1 to 0.3 mμmoles/mg protein) remained in the particles after washing. 3–5 % of the total weight (protein plus lipide) was extractable into hot ethanol and petroleum ether. Thus only a small amount of lipide was associated with the particles. In the best preparations, less than 0.1 mμmole of Coenzyme Q/mg protein was found. Higher levels of Coenzyme Q occasionally were encountered, but were found to be present in those particles having higher than usual levels of cytochrome c_1.

TABLE I

COMPOSITION OF THE SUCCINIC DEHYDROGENASE–CYTOCHROME *b* PARTICLE

Component	*Content**
Heme *b*	3.2–3.4
$c_1 + c$	0.0–0.3
a	0
Trypsin-extractable flavin**	1.6–1.7
Acid-extractable flavin	0
Non-heme iron	6.0–6.4
Lipide	3–5 %

* Units are mμmoles/mg protein unless otherwise noted.

** This flavin was released by acid only after digestion with trypsin.

Enzymic activity

The dehydrogenating activities of the fs(b)$_2$ preparation expressed as μmoles succinate oxidized/min/mg protein with several electron acceptors were as follows: ferricyanide, 3.9; CoQ, 8.9; and 2,6-dichlorophenol–indophenol, 8.0. No cytochrome *c* reductase activity was noted except in some preparations with high residual cytochrome c_1 levels.

The particles catalyzed the rapid reduction of CoQ by succinate only when the assay medium was supplemented with a mitochondrial lipide extract (*cf.* Table II) prepared by the method of BASFORD AND GREEN[14]. The active component in the lipide extract could not be replaced with a wide variety of detergents, purified phospholipides or bovine plasma albumin. The identity of the active component in the lipide extract and the mechanism of its action are under investigation.

The data given in Table II indicate that CoQ is an intermediate electron carrier in the reduction of indophenol by succinate. SINGER *et al.*[15] have established that the succinic flavoprotein does not react directly with indophenol. The succinic-indophenol reductase activity of mitochondria is insensitive to antimycin A which rules out the participation of cytochromes c_1 or c in this reaction. The only two known electron carriers that function between the flavoprotein and cytochrome c_1 are cytochrome *b* and CoQ. In aged fs(b)$_2$ preparations the cytochrome *b* cannot be reduced with succinate but these particles still catalyze the succinic-indophenol reaction as rapidly as a fresh preparation, which indicates that cytochrome *b* is not an essential electron carrier in the reduction of indophenol. It is, of course, possible that indophenol can also react at the same site on the enzyme as CoQ but with a lower turnover rate. In all of the preparations studied so far, the succinic indophenol and CoQ reductase activities were closely associated. A preparation capable of catalyzing the reduction of indophenol but not CoQ was never observed, and in every preparation known to have a relatively low CoQ content, the addition of CoQ to the assay medium stimulated the rate of indophenol reduction.

TABLE II

THE EFFECT OF MITOCHONDRIAL LIPIDES ON THE fs(b)$_2$ CATALYZED REDUCTION OF CoQ AND INDOPHENOL BY SUCCINATE

The rates are expressed as μmoles succinate oxidized/min/mg protein. The assay conditions are described under METHODS.

Additions	*Electron acceptor*	
	CoQ	*Indophenol*
fs(b)$_2$	0.8	2.1
fs(b)$_2$ + Triton X-100	1.8	2.6
fs(b)$_2$ + Triton X-100 + Lipid extract*	8.9	4.7
fs(b)$_2$ + Triton X-100 + Lipid extract + CoQ**	—	8.0

* The lipide extract did not contain CoQ. The SDC was first extracted with acetone to remove CoQ (see ref. 17) and then the lipides were extracted with deoxycholate and butanol as described by BASFORD AND GREEN[14].

With CoQ as the final electron acceptor, 2–20 μg of lipide/ml in the assay medium were required for maximal activity. The exact amount necessary had to be determined empirically for the individual lipide extracts. All of the extracts inhibited the reaction at high concentrations (30–60 μg/ml) and only a few stimulated maximally at 2–5 μg/ml.

** The final concentration of CoQ was 1 mμmole/ml. 5 mμmoles/ml of CoQ inhibited the reduction of indophenol, but the inhibition could be reversed by added 50–100 μg of Triton X-100. Triton did not inhibit the reaction at these levels and the detergent was routinely added to the cuvette to prevent the inhibition by CoQ.

Spectrum

The cytochrome in the fs(b)$_2$ complex was identified as cytochrome *b* by the following criteria: (a) Reduced α, β and Soret peaks were found at 562, 530 and 430 mμ respectively; (b) all the heme in the particle was extractable with acid acetone; (c) the spectrum of the pyridine haemochromagen was indistinguishable from that of protohemin.

60–85 % of the cytochrome *b* in fresh preparations of the particle was reducible by the addition of succinate (Fig. 1). Storing the particle suspension overnight at either 0° or —20° resulted in irreversible loss of cytochrome *b* reduction by succinate together with rapid loss of ferricyanide activity. The ability of the particle to couple succinate oxidation to either CoQ or 2,6-dichlorophenol indophenol reduction, however, was only slightly affected by repeated freezing and thawing or by storage at —20° for several weeks.

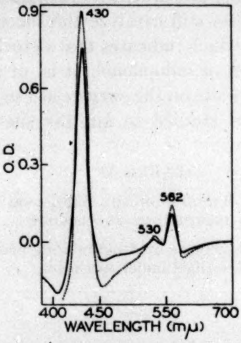

Fig. 1. Difference spectra (reduced minus oxidized) of the succinic dehydrogenase–cytochrome *b* complex recorded with a Beckman DK-2 recording spectrophotometer at room temperature. ———, reduction obtained with succinate; ············, reduction upon addition of dithionite.

DISCUSSION

Although the isolation of a functionally intact succinic dehydrogenase-cytochrome *b* particle has not, to our knowledge, been reported from mammalian sources, such a particle has been prepared from microorganisms[3, 16]. The close association of the succinic flavoprotein and cytochrome *b* in mammalian tissues has, however, been suggested previously[2, 13].

The specific requirement for CoQ in the antimycin A sensitive oxidation of succinate by mitochondria has been demonstrated[17]. The observation of HATEFI[18] and PUMPHREY AND REDFEARN[19] that the reoxidation of reduced CoQ is antimycin A sensitive, placed CoQ before cytochrome c_1 in the terminal electron transport pathway. The rapid reduction of externally added CoQ by the fs(b)$_2$ particle under conditions in which cytochrome *b* is not reducible suggests that the cytochrome is not an obligatory oxido-reduction component in the reduction of the coenzyme by succinate.

When chemically reduced CoQ is added to the SDC preparation of RABINOWITZ AND DE BERNARD[20] which contains cytochrome *b* and c_1 but no *c* or *a*, only cytochrome c_1 is reduced and this reduction is antimycin A sensitive[21]. All attempts to reduce cytochrome *b* by reduced CoQ have been uniformly unsuccessful. The possibility of the interaction of cytochrome *b* and internal CoQ in more complex particles has not been completely eliminated, however.

Previous studies have ruled out cytochrome *b* as an essential component in the reoxidation of reduced CoQ. Since evidence presented in the present study indicated that cytochrome *b* does not participate in the reduction of CoQ by succinate, the existence of a succinic flavoprotein that reduces cytochrome *b* and CoQ by separate pathways seems necessary. The demonstration by CHANCE[1] of the slow rate of cytochrome *b* reduction in non-phosphorylating mitochondrial particles is in accord with this idea. The following formulation for the electron transfer sequence in the present communication would account for the recorded observations:

$$\begin{tikzcd}
 & & \text{Cytochrome } b \\
\text{Succinate} \arrow[r, dashed] & \text{Flavoprotein} \arrow[ur] \arrow[dr] & \\
 & & \text{CoQ}
\end{tikzcd}$$

All attempts to link the soluble succinic dehydrogenase preparation of Singer *et al.*[10,11] to the reduction of either CoQ or 2,6-dichlorophenol–indophenol by succinate have been unsuccessful. A more complex form of the succinic dehydrogenase which reduces CoQ has been isolated in this laboratory and is described in a separate report[22].

ACKNOWLEDGEMENTS

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A NOVEL SYSTEM FOR THE INCORPORATION OF AMINO ACIDS BY EXTRACTS OF *E. COLI* B

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SUMMARY

The incorporation of leucine into an acid insoluble product has been observed in an *in vitro* system obtained from *E. coli*. This system contains a particulate fraction, a soluble fraction and RNA. Only certain amino acids are fixed by this system, which is not inhibited by chloramphenicol or lecithinase A.

Optimal incorporation requires ATP, Mg^{++}, inorganic phosphate, and a sulfhydryl containing compound, and shows characteristics previously undescribed for the incorporation of amino acids.

INTRODUCTION

While investigating amino acid incorporation in both normal and bacteriophage T_2 infected *Escherichia coli* cells, an *in vitro* system, capable of incorporating leucine into an acid insoluble precipitate, was studied. This cell free system has been obtained from T_2 infected cells as well as from uninfected cells. The present paper will discuss this *in vitro* system which requires a soluble fraction, a particulate fraction, and seems to involve RNA. In certain respects the characteristics of this system differ from other amino acid incorporating systems which have been reported[1–4]. It is insensitive to chloramphenicol and lecithinase A, is stimulated by inorganic phosphate and sulfhydryl containing compounds, and seems to incorporate only certain amino acids.

Previous work by BERG *et al.*[1] with a soluble system obtained from *E. coli* has shown the activation and transfer of amino acids onto a ribonucleic acid fraction. The participation of RNA, soluble fractions, and microsomes in amino acid incorporating systems obtained from animal cells has been indicated by the work of ZAMECNIK and his group[2]. NISMAN, using a preparation from lysed protoplasts of *E. coli*, has also demonstrated the activation and incorporation of a number of amino acids[3]. Recently, FUKUHARA AND NISMAN have reported amino acid incorporation by a system obtained from *E. coli* protoplasts[5] which may be similar in part to the one described in this paper.

Abbreviations: RNA, ribonucleic acid; ATP, ADP, AMP, adenosine triphosphate, diphosphate, and monophosphate respectively; UTP and UDP, uridine triphosphate and diphosphate respectively: GTP and GDP, guanosine triphosphate and diphosphate; CTP and CDP, cytidine triphosphate and diphosphate; Tris, tris(hydroxymethyl)-aminomethane; TEA, triethanolamine; TCA, trichloracetic acid, EDTA, ethylenediaminetetraacetic acid; [U-^{14}C], uniformly labelled.

EXPERIMENTAL

Materials

Crystalline ATP, UTP, CTP, GTP, ADP, UDP, GDP, CDP, AMP were purchased from the Sigma Chemical Co.; [8-^{14}C]ATP and AMP from Schwarz Laboratory, Inc.; ribonuclease, deoxyribonuclease, trypsin, and lysozyme from Worthington Biochemical Corp.; lecithinase A was the kind gift of Dr. M. Rodbell[**]. DL-[1-^{14}C]leucine, L-[U-^{14}C]lysine, DL-[1-^{14}C]norleucine, L-[U-^{14}C]phenylalanine, L-[α-^{14}C]histidine, DL-[2-^{14}C]glutamic, DL-[4-^{14}C]aspartic, and DL-[1-^{14}C]alanine were obtained from the Volk Radiochemical Corp.; DL-[1-^{14}C]valine, and L-[1-^{14}C]serine were obtained from Nuclear Chicago Corp.; [1-^{14}C]glycine came from Tracerlab. [^{14}C]BaCO$_3$ and ^{32}P inorganic phosphate were obtained from Oak Ridge National Laboratory. L-[U-^{14}C]proline and DL-[2-^{14}C]tyrosine were obtained from Dr. C. Mitoma[***]. DL-[3-^{14}C]Tryptophan and DL-5-hydroxy-[3-^{14}C]tryptophan were the kind gift of Dr. H. Weissbach[**], γ-[1-^{14}C]amino butyric acid was obtained from Dr. W. Jakoby[**]. D-[U-^{14}C]Glucose was purchased from New England Nuclear Corp. [1-^{14}C]Ribose-5-phosphate was prepared by the method of Horecker *et al.*[6]. Soluble RNA was prepared by a hot detergent extraction of *E. coli* cells following the procedure of Crestfield *et al.*[7], und using that RNA which is not precipitated by 1 *M* NaCl (soluble RNA). The L-amino acid mixture used was 0.02 *M* with respect to the following: threonine, tyrosine, phenylalanine, methionine, cystine, isoleucine, glutamine, glutamic acid, aspartic acid, histidine, hydroxyproline, lysine, proline, alanine, glycine, tryptophan, asparagine, serine, arginine, and valine. In some experiments the equilibrated mixture described by Nisman[3] was employed.

METHODS

Protein was determined by the method of Lowry *et al.*[8]. ^{14}C activity was measured in a Nuclear Chicago Automatic thin window gas flow counter, model D-47. U.V. absorption measurements were made with a Beckman DU Spectrophotometer.

Isolation of soluble fraction and particles

E. coli was grown at 37° on a minimal medium[9] containing 0.5 % glucose. Cultures were harvested during the logarithmic phase of growth when the cell concentration had reached $1 \cdot 10^9$ cells per ml. After washing once with distilled water the cells were suspended in 3 times their weight of cold 0.01 *M* Tris–succinate buffer[*] at pH 7.5, containing 0.005 *M* MgCl$_2$ (Tris–succinate–Mg^{++}), and were broken by sonic oscillation for 15 min in a Raytheon 10 kc Sonic Oscillator. The broken cell suspension was centrifuged at 20,000 × *g* for 20 min and the residue was discarded. The supernatant fluid was centrifuged at 105,000 × *g* for 3 h in a Spinco Model L ultracentrifuge. The pellet obtained after the 105,000 × *g* centrifugation was resuspended in the Tris–succinate–Mg^{++} buffer and recentrifuged at 105,000 × *g* for 3 h. The supernatant fluid was decanted and the pellet was suspended in Tris–succinate–Mg^{++} buffer to achieve a concentration of 30 mg of protein/ml and stored at −18°. This material represents the "particulate" fraction. The supernatant from the first 105,000 × g centrifugation was dialyzed overnight against running Tris–succinate–Mg^{++} buffer using a rocking dialyzer. The dialyzed supernatant fluid represents the "soluble" fraction.

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[*] Suggested by Drs. Roberts and Bolton of the Carnegie Institute of Washington.

[**] National Institutes of Health, Bethesda, Md.

[***] Stanford Research Institute, Menlo Park, Calif.

Preparation of T_2 bacteriophage: The method of HERRIOT AND BARLOW was used[11].

Assay of amino acid incorporation

After incubation, 3 ml of 5 % perchloric acid and 1.5 ml of a Celite suspension (2 mg/ml) were added to the incubation mixtures at 0°. The mixture was stirred manually, filtered on a Millipore filter (HA, 1 inch diameter) and the precipitate was washed twice with 5-ml portions of cold 0.2 N perchloric acid and once with 5 ml of cold 0.002 N perchloric acid. The filter was dried in air and then placed on a copper planchet for counting.

RESULTS

Requirements of the system

Washed particles obtained from *E. coli* are unable to incorporate amino acids into an acid insoluble product unless some "soluble" fraction is present. Though this system will incorporate certain other amino acids, most of the work was done with leucine as the radioactive substrate.

The maximal incorporation of leucine by washed particles requires ATP, inorganic phosphate and a sulfhydryl compound in addition to a soluble, non-dialyzable fraction (Table I). The requirements of the system are the same in the absence of exogenous soluble-RNA (Table I) or in the presence of added soluble RNA (Table II). However, the addition of soluble RNA markedly increases incorporation in this system as shown in Fig. 1. It can be noted (Tables I and II) that heating the soluble fraction at 100° for 5 min destroys the leucine incorporation system almost completely, whereas the particles retain 60 to 70 % of their activity after heating under the same conditions.

The limited ability of the heated particles to incorporate leucine may be explained

TABLE I

INCORPORATION OF [^{14}C]LEUCINE IN THE ABSENCE OF SOLUBLE RNA

Complete system contained 50 µmoles TEA buffer, pH 8.2, 144 µmoles mercaptoethanol, 4 µmoles potassium phosphate buffer, pH 8.0, 1.7 µmoles of ATP, 0.06 µmoles of DL-[1-^{14}C]leucine containing $3 \cdot 10^5$ counts/min, 0.05 ml of particles (in 0.01 M Tris–succinate buffer containing 0.005 M $MgCl_2$) containing 30 mg protein/ml, and 0.6 mg of soluble fraction protein in a total volume of 0.60 ml. Incubations were carried out at 37° for 60 min.

Condition	*Counts/min fixed**
Optimal	1910
No ATP	296
No $PO_4^{=}$	1580
No mercaptoethanol	72
Using heated particles	1190
Using heated soluble fraction	50
No particles	55
No soluble fraction	27

* In the acid insoluble precipitate.

TABLE II

INCORPORATION OF [^{14}C]LEUCINE IN THE PRESENCE OF SOLUBLE RNA

Complete system was the same as in Table I plus 5 O.D. units of soluble RNA. This amount of RNA stimulates the incorporation of leucine about 50 % under these conditions.

Conditions	*Counts/min fixed*
Optimal	2820
No soluble fraction	74
No particles	1660
Using heated particles	1925
Using heated soluble fraction	142
No ATP	224
No $PO_4^=$	2030
Using heated particles and heated soluble fraction	43
No mercaptoethanol	112

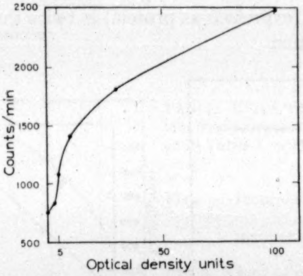

Fig. 1. Effect of soluble RNA upon leucine incorporation. System was the same as Table I, with a total incubation volume of 0.75 ml.

if the particles contain a heat stable ribonucleic acid fraction which is capable of accepting amino acids, as described in BERG's system[1]. The fact that the particles do lose 30–40 % of their incorporating power after heating indicates that they function as more than a RNA carrier. This is supported by other data presented in this paper in which it will be shown that the product of leucine incorporation does not, for the most part, consist of a RNA-amino acid linkage.

It should also be noted that the concentration of sulfhydryl compound, in this case mercaptoethanol, necessary for maximal incorporation, is of the order of 0.25–0.30 *M*.

Inhibition by EDTA

EDTA completely inhibits incorporation at a concentration of $5 \cdot 10^{-3}$ *M*; this is reversed by Mg^{++} or Co^{++} as shown in Table III. Mn^{++} is much less effective in restoring activity and, at a concentration of 10^{-2} *M*, inhibits incorporation 50 % even in the presence of Mg^{++}. Since the particles and the soluble fraction were always stored in a Tris–succinate buffer which contained Mg^{++}, it was found unnecessary to add further Mg^{++} to the incubation mixtures.

TABLE III

REQUIREMENT FOR A METAL ION

Incubations were the same as in Table I, except that the total volume of incubation was 0.70 ml, and 1.0 mg of particles were used.

Additions	*Counts/min fixed*
None	552
2 µmoles EDTA	12
2 µmoles EDTA + 5 µmoles Mg^{++}	530
2 µmoles EDTA + 5 µmoles CO^{++}	550
2 µmoles EDTA + 5 µmoles Mn^{++}	350
2 µmoles EDTA + 5 µmoles Fe^{+++}	186

Effect of enzyme concentrations

The fixation of leucine as a function of the concentration of the particles or of the soluble fraction is shown in Figs. 2 and 3. Maximal incorporation occurs when the concentration of particles (expressed as protein) is twice that of the protein concentration of the soluble fraction.

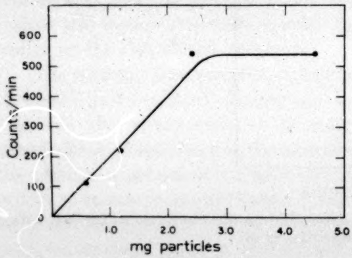

Fig. 2. Effect of particle concentration upon leucine incorporation. System used was the same as in Table I, with a total incubation volume of 0.70 ml.

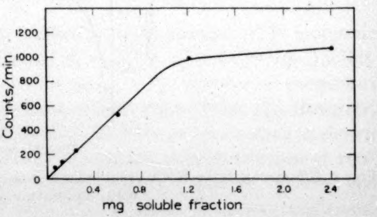

Fig. 3. Effect of soluble fraction upon leucine incorporation. System used was the same as in Table I, with a total incubation volume of 0.70 ml.

Effect of leucine concentration

The effect of the leucine concentration upon leucine incorporation is shown in Fig. 4. In the experiments reported in this paper, the leucine concentration used was 0.06 µmoles per incubation mixture of 0.6 ml.

Kinetics of incorporation

The time course of leucine incorporation is shown in Fig. 5. After one hour at 37° the rate of incorporation decreased markedly and, hence, incubations were carried out for 60 min. In the early part of the work a lag period of 30 to 45 min was noticed before leucine incorporation proceeded at an appreciable rate. The lag period could be abolished by the addition of low concentrations of inorganic phosphate. The ratio of inorganic phosphate to ATP was also found to be important since concentrations greater than 0.010 *M* of either or both reactants are inhibitory to leucine incorporation.

pH effect

The optimal pH for leucine incorporation is 8.2 (Fig. 6) although incorporation is observed over a wide pH range. Below pH 7 there is little or no incorporation. The active incorporation of leucine at relatively high pH's, *i.e.* pH 8–10, is of interest in light of BERG's finding[1] that the amino acid–RNA complex formed by the *E. coli* system he used is unstable above pH 7.0.

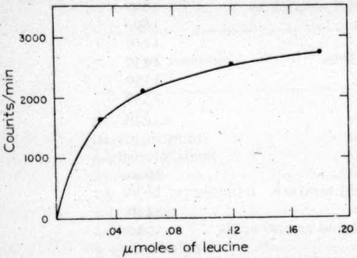

Fig. 4.

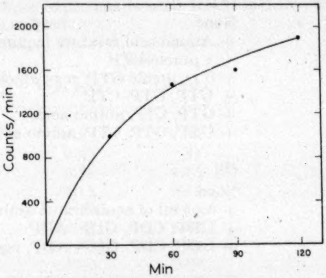

Fig. 5.

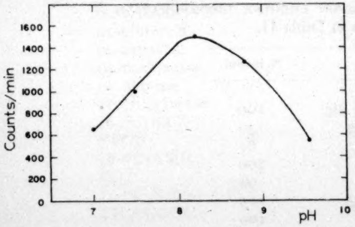

Fig. 6.

Fig. 4. Effect of leucine concentration upon leucine incorporation. System used was the same as in Table I, with a total incubation volume of 0.70 ml.

Fig. 5. Incorporation of leucine with time. System was the same as in Table I, with a total incubation volume of 0.50 ml.

Fig. 6. Effect of pH upon leucine incorporation. System was the same as in Table I, with a total incubation volume of 0.50 ml. Tris buffer (50 μmoles) was used in all cases up to pH 8.85; above that pH a carbonate buffer was used.

Effect of nucleotides

In systems derived from animal cells, GTP and CTP have been reported to be involved in protein systhesis[2]. NISMAN has reported a large stimulation of amino acid incorporation, in *E. coli* extracts, with a mixture of nucleoside diphosphates[3]. Nucleoside mono- and diphosphates seem to have no effect upon amino acid incorporation in the system described here, and the nucleoside triphosphates, other than ATP, are slightly inhibitory. This is particularly so in the case of UTP (Table IV). The inhibition by UTP, is accentuated by the presence of an equimolar mixture of 20 amino acids (lacking leucine) although the amino acid mixture itself had no effect upon leucine incorporation in the absence of UTP.

Effect of antibiotics and other reagents

The effect of various other reagents upon leucine incorporation is summarized in Table V. Although chloramphenicol is reported to be a powerful inhibitor of protein synthesis[12], it has no influence upon leucine incorporation in this system. Indeed, none of the antibiotics were effective inhibitors although streptomycin was somewhat

TABLE IV

EFFECT OF NUCLEOTIDES AND AMINO ACIDS UPON LEUCINE INCORPORATION

(A) Incubations as in Table II, using 1 mg of particles. (B) Incubations as in Table II. 0.30 μmole of each nucleotide were used in the incubations.

Additions	*Counts/min fixed*
(A)	
None	1800
+ Amino acid mixture (equimolar)	1800
+ 1 μmole UTP	1210
+ 0.15 μmole GTP, 0.25 μmole CTP	1450
+ UTP, GTP, CTP	1150
+ GTP, CTP, amino acids	1550
+ UTP, GTP, CTP, amino acids	550
(B)	
None	2640
+ 0.05 ml of equilibrated amino acid mixture	2670
+ UDP, CDP, GDP, ADP	2440
+ UDP, CDP, GDP, ADP, equilibrated amino acids	2730

TABLE V

EFFECT OF VARIOUS REAGENTS UPON LEUCINE INCORPORATION

Incubations as in Table II.

Reagent	*% control*
Chloramphenicol, 0.25 mg	100
Streptomycin, 0.25 mg	70
Terramycin, 0.25 mg	81
Penicillin, 0.25 mg	100
Thyroxine, 0.01 mg	96
Testosterone, 0.004 mg	105
Arsenate, 8 μmoles	100
Arsenite, 5 μmoles	91
Dinitrophenol, 5 μmoles	83
Colchicine, 0.5 μmole	107
B_{12}, 0.20 μg	100
Deoxycholate, 2.5 mg	0
Cyanide, 5 μmoles	105

inhibitory. The latter observation may be due to the known affinity of streptomycin for RNA. Of the various reagents tested only deoxycholate (2.5 mg per incubation mixture) completely destroyed incorporation, presumably by destroying the structure of the particles. Solubilization of the particles could be seen immediately upon the addition of deoxycholate. Arsenate did not inhibit leucine incorporation in the presence of phosphate.

Incorporation of other amino acids

The ability of the system to incorporate other amino acids or substrates is shown in Table VI. Of the amino acids tested only leucine, tryptophan, serine, 5-hydroxy tryptophan, histidine and lysine were incorporated to a significant extent whether or not exogenous soluble RNA was present. Of these only leucine incorporation is markedly stimulated by soluble RNA. The significance of the small incorporation of glucose, ATP, and inorganic phosphate is not known.

TABLE VI

INCORPORATION OF DIFFERENT AMINO ACIDS

Incubations as in Table I. About $3 \cdot 10^5$ counts/min of each radioactive compound was placed in the incubation mixture. In the case of the amino acids the "% fixed" was calculated assuming that only the L isomer was incorporated. In those experiments with RNA, 5 O.D. units of soluble RNA was added.

^{14}C compounds	*µmoles added*	*% fixed*	
		No RNA	*+ RNA*
DL-leucine	0.06	0.25	0.80
DL-tryptophan	0.06	0.37	0.37
L-phenylalanine	0.06	0.30	0.25
DL-serine	0.06	0.15	0.15
DL-5-OH tryptophan	0.10	0.10	0.15
L-histidine	0.06	0.07	0.05
L-lysine	0.06	0.05	0.04
γ-aminobutyric	0.10	0.02	0.02
DL-valine	0.06	< 0.01	0.01
L-proline	0.06	< 0.01	0.01
glycine	0.06	< 0.01	0.01
DL-tyrosine	0.06	< 0.01	0.01
DL-glutamic	0.06	< 0.01	0.01
DL-aspartic	0.06	< 0.01	0.01
DL-norleucine	0.06	< 0.01	0.01
DL-alanine	0.06	0.02	0.02
[U-^{14}C]glucose	0.10	—	0.05
[8-^{14}C]ATP	0.10	—	0.05
$^{32}PO_4^{=}$	0.10	—	0.05
[8-^{14}C]AMP	0.10	—	< 0.01
$CO_3^{=}$	0.25	—	< 0.01
[1-^{14}C]Ribose-5-PO_4	0.20	—	< 0.01
$CH_3{}^{14}COOH$	0.20	—	< 0.01

Effect of hydrolytic enzymes upon formation of acid insoluble product containing leucine

The nature of the acid insoluble, nondialyzable product which is formed in these incubation mixtures and contains [^{14}C]leucine remains unclear. Table VII shows the effect of various hydrolytic enzymes upon the product. Ribonuclease, when present during the incubation prevents leucine incorporation, though when it is added after the incubation only 30 % of the product is destroyed. Trypsin, on the other hand, when added before or after the incubation period completely destroys any acid insoluble product containing leucine. The other enzymes tested had no effect upon leucine incorporation.

Stability of acid insoluble product towards heat, acid and alkali

The stability of the acid insoluble product formed in these incubations is shown in Table VIII. Heating the incubation mixtures for a short time, after the regular incubation period of 1 h, in the presence of TCA or ethanol, increases the leucine content of the acid insoluble product by a, presumably, non-enzymic reaction. On the other hand, heating the incubation mixtures in 1 *N* NaOH or in 1 *N* HCl at 100°,

TABLE VII

STABILITY OF INCORPORATION PRODUCT TOWARDS VARIOUS ENZYMES

Incubations as in Table II. The enzymes tested were added either at the beginning of the incubation and were present throughout the incubation period, or were added after 60 min and the incubation continued for 30 more minutes. In the latter case the results were compared to a control run for 90 min.

Addition		% of ^{14}C incorporated by control Added during incubation	Added after incubation
Ribonuclease	0.02 mg	0	70
Trypsin	0.10 mg	0	0
Desoxyribonuclease	0.02 mg	100	100
Lysozyme	0.20 mg	95	100
Crude snake venom	0.10 mg	76	
Lecithinase A	0.05 mg	100	

TABLE VIII

STABILITY OF PRODUCT FORMED WITH [^{14}C]LEUCINE AS PRECURSOR

Incubations as in Table I. Additions were made at the end of a 1 h incubation as follows: 1) 3 ml of 5 % TCA added; 2) and 3) 5 ml of ethanol added; 4) and 5) 5 ml of chloroform added; 6) 1 ml of 1.0 *N* NaOH added; 7) and 8) 1 ml of 1.0 *N* HCl added. The acid insoluble precipitates were collected and counted as described in the experimental section. In the case of 6) 1 ml of 1.0 *N* HCl was added to the incubation before addition of perchloric acid.

Expt.	Treatment	% control
1	5 % TCA, 100°, 10 min	150
2	Ethanol, 5 min	100
3	Ethanol, 80°, 5 min	150
4	$CHCl_3$, 5 min	100
5	$CHCl_3$, 61°, 5 min	100
6	1 *N* NaOH, 100°, 10 min	10
7	1 *N* HCl, 100°, 10 min	36
8	1 *N* HCl, 100°, 45 min	20

after the regular incubation period, leads to a rapid loss of acid precipitable material containing ^{14}C. This effect seems much more pronounced in the alkali degradation.

In one experiment the acid precipitable material formed after incubation with [^{14}C]leucine was collected, washed thoroughly, and subjected to hydrolysis in 6 *N* HCl at 100° for 18 h. The hydrolysate, following removal of HCl *in vacuo*, was chromatographed on paper using phenol saturated with water, acetone–water (85:15, v/v) and butanol–propionic acid–water (125-62-88, v/v) as the developing solvents. 75 % of the radioactivity present in the hydrolysate moved with the same R_F as authentic leucine and about 25 % of the ^{14}C was associated with a slower moving material which was dark brown, insoluble in water, and probably represented a humin like material.

In vitro *incorporation by normal and* T_2 *infected cells*

The incorporation of amino acids by *E. coli* cells, *in vivo*, diminishes markedly in the latter part of the latent period after infection with T_2 bacteriophage. This is shown in Table IX which summarizes the incorporation of [^{14}C]glycine and leucine as a function of time after infection. Ten to twenty minutes after T_2 infection, the *E. coli* cells incorporate amino acids at about one-half the rate of uninfected cells. It was of interest, therefore, to investigate the leucine incorporating systems obtained from such infected cells. The *in vitro* leucine incorporating system as described in this paper was obtained from normal *E. coli* B cells. This system can also be obtained from T_2 bacteriophage infected cells up to the time of lysis, and it was found that there was no significant loss or gain of leucine incorporating ability of either the particles or the soluble fraction, upon T_2 infection. This is also true for the system which activates and transfers amino acids to RNA[1]. T_2 infection does not seem to alter the ability of the soluble RNA to accept leucine, nor does it affect the activating enzyme for this amino acid. However, the particles sedimentable at 105,000 × *g* which are obtained from T_2 infected cells are less stable than those obtained from normal cells, and rapidly clump and precipitate upon standing. In addition, particles obtained from phage infected cells have a relatively lower nucleic acid content as measured by the ratio of absorbance at 280 mμ to 260 mμ (Table X).

TABLE IX

INCORPORATION OF ^{14}C AMINO ACIDS BY *E. coli* B

(A) 1 l of *E. coli* in the logarithmic growth phase was given 67 μmole of [^{14}C]glycine containing $1 \cdot 10^7$ counts/min for the stated interval. At the end of this time the cell suspension was cooled to 0–5° within 2 min and the cells were centrifuged down and washed twice with cold water at 0°. The cells were suspended in 5 times their weight of 0.01 *M* Tris–succinate–Mg^{++} buffer and disrupted in a 10 kc Raytheon Sonicator for 15 min. The broken cell suspension was centrifuged at 20,000 × *g* for 20 min and the supernatant was dialyzed for 24 h against 0.01 *M* Tris–succinate–Mg^{++} buffer. An aliquot of the dialyzed solution was counted. (B) As A, but the cells were exposed to 2.6 μmoles of leucine containing $1.8 \cdot 10^7$ counts/min.

Time of exposure to amino acid after T_2 phage infection	*Counts fixed per liter*
(A)	*[1-^{14}C]glycine*
No infection (10 min)	$1.6 \cdot 10^6$
0–10 min	$1.7 \cdot 10^6$
10–20 min	$0.9 \cdot 10^6$
20–30 min	$0.7 \cdot 10^6$
(B)	DL-*[1-^{14}C]leucine*
No infection (3 min)	$5.2 \cdot 10^6$
15–18 min	$2.6 \cdot 10^6$

TABLE X

280/260 RATIO OF PARTICLES FROM *E. coli*

At stated intervals after T_2 infection, the *E. coli* cells were rapidly cooled to 0° and particles were isolated as described in the experimental section. Two separate experiments are shown.

Time after T_2 infection (min)	*$\frac{280^*}{260}$*	
0	0.63	0.60
10	0.69	0.65
20	0.74	0.69

* Ratio of absorbance at 280 mμ to 260 mμ.

DISCUSSION

Previous work has suggested that the incorporation of an amino acid into protein may involve activation of the amino acid, transfer of the activated amino acid onto a ribonucleic acid molecule, transfer of the amino acid from the ribonucleic acid molecule to a ribonucleoprotein, and eventual incorporation into the finished protein[2]. In some aspects, the data reported here is compatible with such a mechanism. There is a requirement for ATP, a soluble and particulate fraction, and RNA for the maximal incorporation of leucine in this system. The particles also possess a heat stable molecule capable of accepting leucine in the presence of the appropriate enzymes. This heat stable substance may be a RNA molecule since these particles are known to have a high RNA content[10]. Also, the incorporation of leucine by this system is stimulated by the addition of soluble RNA as would be expected if the enzymes catalyzing the transfer of amino acids to RNA[1] were present. In this respect it should be noted that leucine incorporation is observed at pH's up to 10 which is far above the optimal pH of 7.0 described by BERG[1], and is in a pH range at which BERG found the amino acid–RNA complex to be quite unstable.

It is unlikely that the final product containing leucine which is formed by the complete, unheated, system contains a leucine–RNA bond. Though ribonuclease destroys the ability of the system to incorporate leucine, once incorporation has occurred, ribonuclease is unable to release most of the bound amino acid. Coupled with the observation that trypsin completely destroys the product of leucine incorporation, either during or after incubation, points to a final product which contains a major fraction of the leucine in peptide linkage with, perhaps, some bound to a RNA molecule. This is supported by the isolation of leucine from acid hydrolysis of the product.

In other respects, the leucine incorporating system described in this paper has characteristics which are difficult to reconcile with present concepts of protein synthesis. Chloramphenicol, reported to have a strong inhibitory action upon protein synthesis, has no effect in this system. The addition of other amino acids which would be expected to be necessary for protein synthesis, had no stimulatory effect upon the incorporation of leucine. In addition, only certain amino acids seemed to be fixed and, of these, only leucine incorporation was stimulated by soluble RNA. Since NISMAN has reported that many of the activating enzymes in *E. coli* are unstable to sonication[3] the inability to observe the incorporation of many of the amino acids may be due to this factor. Another possible explanation is that DL-[^{14}C]amino acids were used as substrates in many cases (Table VI), and one optical isomer may be inhibiting utilization of its enantiomorph, as described for the amino acid activating system in *E. coli*[3]. Finally, the role of inorganic phosphate in stimulating the incorporation of leucine is unexplained. Phosphate stimulation is not inhibited by the presence of arsenate and there also seems to be a small incorporation of phosphate into the acid precipitable product formed in the incubation mixtures containing leucine.

Though the significance of the incorporation of leucine in the system remains unknown, it may represent some early phase of peptide or protein synthesis which is not sensitive to chloramphenicol and which does not require a full complement of amino acids.

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NOUVELLE DÉTERMINATION DU POTENTIEL D'OXYDORÉDUCTION DU SYSTÈME LACTATE–PYRUVATE

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SUMMARY

A new determination of the redox potential of the lactate–pyruvate system

For the redox potential, measured in the presence of riboflavine and the D-lactic dehydrogenase of yeast, for the equilibrium D-lactate ⇌ pyruvate at pH 7 and 27°, the value of $E'_0 = -0.190$ V was found. This value is in good agreement with the one estimated by Burton and Wilson. It differs markedly from the earlier values determined without considering the specificity of the enzymes.

INTRODUCTION

Le potentiel du système lactate–pyruvate a été déterminé il y a 25 ans environ presque simultanément dans plusieurs laboratoires, en particulier par Wurmser *et al.*[1,2], Banga *et al.*[3], puis Barron et Hastings[4].

La méthode utilisée était la mesure directe du potentiel d'une électrode de platine plongée dans un mélange lactate–pyruvate à un pH bien défini, en présence d'un colorant électroactif convenable, sous vide. L'équilibre s'établissait lorsqu'on ajoutait une lacticodéshydrogénase sous la forme d'un extrait à peine purifié de bacille *Coli*, de muscle cardiaque ou de gonocoques, suivant les cas.

La relation entre le potentiel mesuré de l'électrode de platine E et le potentiel de demi-réduction à pH 7 du système lactate–pyruvate E'_0 est:

$$E = E'_0 - \frac{RT/2f}{\log_{10} e} \log_{10} \frac{(\text{lactate})}{(\text{pyruvate})} - \frac{RT/f}{\log_{10} e} (\text{pH-7})$$

Ces déterminations ont donné pour E'_0 les valeurs —178 mV à 37°[2], —181 mV à 37°[3] et —180 mV à 35°[4], auxquelles correspondent des valeurs de ΔF^0 approximativement égales à 11.5 kcal.

Burton et Wilson[5] ont contesté l'exactitude de ces valeurs: ils ont en effet déterminé avec précision, par plusieurs méthodes, le potentiel du système DPN–DPNH. A partir de leurs résultats et des données de Racker[6] et de Kubowitz et Ott[7] sur la constante d'équilibre de la réaction

$$\text{pyruvate} + \text{DPNH} \xrightleftharpoons[\text{déshydrogénase}]{\text{L-lactico-}} \text{L-lactate} + \text{DPN}$$

ils ont calculé que la valeur de E'_0 pour le système lactate–pyruvate est —190 mV à 25°, (correspondant à $\Delta F^0 = 10.32$ kcal).

D'après Burton et Wilson, les différences entre leurs déterminations et les valeurs potentiométriques s'expliqueraient par le fait que l'équilibre thermodynamique n'était pas atteint dans les mesures de potentiel.

En réalité, il faut considérer que les mesures potentiométriques de 1932 ont été interprétées en prenant pour concentration de lactate actif la concentration de lactate total. On ne savait pas, à cette époque, que les lacticodéshydrogénases sont stéréospécifiques et qu'un seul des stéréoisomères du lactate est en équilibre avec le pyruvate. C'est seulement en 1943[7] qu'a été mise en évidence la stéréospécificité de la lacticodéshydrogénase du muscle pour le L-lactate. Le bacille *Coli* contient les deux D- et L-lacticodéshydrogénases, mais les extraits possèdent l'une ou l'autre suivant la façon dont ils sont obtenus[8]. Une diminution d'un facteur 2 sur la concentration de lactate donne un potentiel trop élevé de 30 log 2 soit 10 mV. Mais il n'est pas possible de faire des corrections précises sur les mesures anciennes puisque l'on ne connaît ni les enzymes utilisés, ni la composition stéréoisomérique exacte des lactates employés.

De nouvelles déterminations ont donc été entreprises et font l'objet du présent travail. On a mesuré potentiométriquement le potentiel lactate–pyruvate, en utilisant un D-lactate pur et la D-lacticodéshydrogénase de la levure[9,10]. Cet enzyme permet, en absence de DPN, l'échange direct des électrons entre le D-lactate ou le pyruvate d'une part et un accepteur électroactif d'autre part. La riboflavine convient particulièrement bien comme accepteur puisque son potentiel de demi-réduction à pH 7 est —208 mV[11,12], donc très voisin de celui du système étudié.

MATÉRIEL ET MÉTHODES

Acide DL-*lactique* pur p. analyse: Hopkins et Williams. Solution 0.5 *M* délactonisée par chauffage en milieu alcalin (NaOH) puis amenée à pH 7.2.

D-*lactate de sodium* préparé à partir de l'acide précédent, selon la technique décrite par ailleurs[10], contenant moins de 1 % de l'isomère L-. La concentration des solutions est mesurée enzymatiquement avec la D-lacticodéshydrogénase de la levure[10].

Pyruvate de sodium (Roche). Les solutions 16.5 mg/ml, soit 0.15 *M*, sont préparées immédiatement avant leur utilisation.

Riboflavine (General Biochemicals). Solutions 0.36 mg/ml, soit 10^{-3} *M*, conservées congelées.

Tampon phosphate 0.1 *M*, pH environ 7, préparé selon Clark et mesuré à l'électrode d'hydrogène.

D-*lacticodéshydrogénase*. Préparée à partir de la levure cultivée en anaérobiose par la méthode de SLONIMSKI *et al.*[13]. Une unité d'enzyme est la quantité qui donne en présence de ferricyanure $10^{-3}\,M$ et de DL-lactate $1.6 \cdot 10^{-2}\,M$ une réduction du ferricyanure correspondant à une variation de densité optique de 0.001/min à 420 mμ et 27°. Cet enzyme, conservé à 27° en présence de lactate dans les conditions expérimentales utilisées, perd en 24 h moins de la moitié de son activité.

Préparation des tubes Thunberg pour l'étude colorimétrique de la réversibilité

On utilise des tubes Thunberg à diverticule, soudés à des cuves Beckman. On traite simultanément deux séries de tubes. Dans la première, les réactifs DL-lactate, riboflavine et pyruvate sont introduits dans un même compartiment; la réaction débute lorsque le contenu de ce compartiment est mélangé avec l'enzyme contenu dans le compartiment secondaire. Dans la seconde série de tubes, on introduit dans le compartiment principal le DL-lactate, la riboflavine et l'enzyme; le pyruvate, placé dans le compartiment secondaire, n'est mélangé qu'après réduction presque complète de la riboflavine. Bien entendu, ces tubes sont privés d'oxygène très soigneusement suivant la technique classique (voir ci-dessous). Ces expériences ont été faites dans les conditions suivantes: tampon phosphate 0.03 M, pH 7.2, riboflavine $4.6 \cdot 10^{-5}\,M$, DL-lactate $6 \cdot 10^{-3}\,M$ ou zéro, pyruvate zéro ou $0.3 \cdot 10^{-3}\,M$ ou $3 \cdot 10^{-3}\,M$ ou $15 \cdot 10^{-3}\,M$. Enzyme: 1000 U.B.

Préparation des tubes Thunberg pour la détermination du potentiel

Nous utilisons des tubes Thunberg à deux compartiments modifiés pour les mesures électrométriques selon BORSOOK ET SCHOTT[14]: la partie inférieure du compartiment principal est prolongée par un capillaire qu'on remplit d'un gel d'agar (3.5 %) contenant 40 % de KCl. Deux électrodes de platine, soudées au bouchon rodé, plongent jusqu'au fond du compartiment principal.

Pour la plupart des expériences, on met dans le compartiment principal les réactifs suivants aux concentrations finales indiquées: tampon phosphate 0.03 M, pH 7; riboflavine $4.6 \cdot 10^{-5}\,M$; D-lactate de sodium $5 \cdot 10^{-3}\,M$; pyruvate 1.55, 2.5 ou $5 \cdot 10^{-3}\,M$. Dans le compartiment secondaire, on met une quantité de D-lacticodéshydrogénase correspondant à environ 1000 unités (voir plus haut).

Dans quelques expériences, on a ajouté le pyruvate au mélange des autres réactifs au moment où le potentiel était déjà descendu à une valeur notablement plus faible que la valeur prévue pour l'équilibre. Le pyruvate était placé dans le compartiment secondaire, et l'enzyme dans le compartiment principal avec les autres réactifs. Un dégazage préliminaire avant l'addition de l'enzyme permet alors d'éviter le moussage lors du vide définitif.

Mesure du potentiel

Une fois les réactifs introduits dans les tubes Thunberg, on passe à la flamme les électrodes de platine, solidaires du bouchon, on ferme, on fait un vide préliminaire à la trompe à eau et on laisse dégazer 15 min. Les tubes sont ensuite vidés puis remplis d'argon à trois reprises, et laissés finalement sous vide. Ils sont placés dans un bain thermostaté à 27°. Le capillaire terminal plonge dans une solution de KCl saturée liée par un pont agar à l'électrode au calomel. Celle-ci est maintenue constamment dans le thermostat à 27°.

Les deux électrodes platine et calomel sont branchées sur un potentiomètre à pont Radiometer PHM 31. Le potentiel est donc mesuré par une méthode d'opposition après standardisation avec la pile Weston. Chaque mesure est faite successivement deux fois: avant et après une légère agitation. Lorsqu'on trouve une différence notable, on ne tient pas compte du résultat.

Nous avons utilisé deux électrodes au calomel à KCl saturé. Ces électrodes ont été étalonnées en mesurant à 25° le potentiel des piles: électrode au calomel/électrode à hydrogène en présence de biphtallate de potassium 0.05 *M* ou d'un tampon acide acétique–acétate de sodium (1:1). La valeur trouvée est $E_c = -243$ mV à 25°.

Mesure du pH

Une fois les mesures de potentiel terminées, on prélève les solutions contenues dans les tubes Thunberg et on détermine à 20° leur pH à l'électrode de verre par rapport à un tampon phosphate étalon. Celui-ci a été mesuré au moyen d'une électrode d'hydrogène avec l'électrode au calomel utilisée pour la mesure du potentiel.

PARTIE EXPÉRIMENTALE

Etude de la réversibilité

On sait[10] que l'enzyme utilisé catalyse l'oxydation du D-lactate par le ferricyanure à raison de 2 molécules de ferricyanure par molécule de lactate. Le pyruvate est donc le produit formé. Il a paru néanmoins utile de s'assurer que, dans nos conditions expérimentales, la réaction peut évoluer facilement dans les deux sens et se stabilise rapidement au même palier. La Fig. 1 montre que la riboflavine réduite en présence

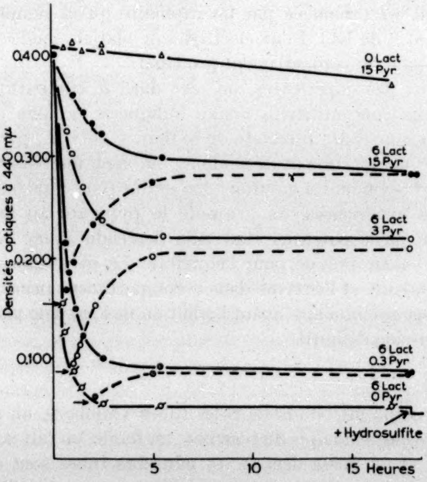

Fig. 1. Etude colorimétrique de la réversibilité de la réaction:

$$\text{D-Lactate} + \text{Riboflavine ox.} \xrightleftharpoons[\text{déshydrogénase}]{\text{D-lactico-}} \text{Pyruvate} + \text{Riboflavine réd.}$$

Tp phosphate 0.03 *M*, riboflavine $4.6 \cdot 10^{-5}$ *M*, enzyme 1000 U.B., D-lactate et pyruvate, concentrations variables indiquées sur la figure en m*M*. Le pyruvate est mélangé aux autres réactifs soit au temps zéro (trait plein), soit aux temps indiqués par une flèche (pointillé).

de lactate seul, se réoxyde lorsqu'on ajoute du pyruvate et que la réaction est parfaitement réversible.

En absence de lactate, le pyruvate ne réduit pas la riboflavine: un témoin sans lactate, contenant $15 \cdot 10^{-3}\,M$ de pyruvate n'a montré en 18 h qu'une décoloration négligeable de la riboflavine. La préparation enzymatique utilisée ne contient donc pas de pyruvate oxydase.

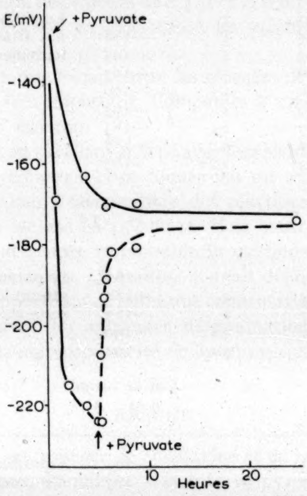

Fig. 2. Evolution du potentiel des électrodes de platine jusqu'à l'équilibre. Tp phosphate 0.03 M, riboflavine $4.6 \cdot 10^{-5}\,M$, D-lactate $5 \cdot 10^{-3}\,M$, enzyme 1000 U.B. Le pyruvate ($5 \cdot 10^{-3}\,M$) est mélangé aux autres réactifs, soit au temps zéro (trait plein), soit au moment indiqué par la flèche (pointillé).

Détermination du potentiel de demi-réduction

Une série de déterminations ont été faites à 27°, pour différentes valeurs du rapport (red)/(ox), à des pH voisins de 7, mesurés chaque fois. La concentration de D-lactate est maintenue constante et égale à $5 \cdot 10^{-3}\,M$, la concentration de pyruvate variant de 1.55 à $5 \cdot 10^{-3}\,M$. L'enzyme est ajouté en quantité suffisante pour que l'équilibre de la réaction s'établisse rapidement; celui-ci est atteint généralement en moins d'une heure. L'échange d'électrons entre le colorant et les électrodes de platine se fait plus ou moins rapidement. Il peut en résulter un certain retard dans l'évolution du potentiel des électrodes par rapport au potentiel réel. Toutefois, la Fig. 2 montre que le potentiel atteint par les électrodes se stabilise correctement et ne dépend pas du sens dans lequel évolue la réaction avant d'atteindre l'équilibre.

Dans les meilleurs expériences, pour lesquelles les électrodes ne présentaient pas du tout d'inertie, le potentiel mesuré atteignait l'équilibre en 1 h et restait constant une vingtaine d'heures. Dans d'autres, le potentiel mesuré descendait lentement, en plusieurs heures, de sorte qu'il était plus difficile de déterminer avec certitude la valeur correspondant au palier. Seules ont été retenues les déterminations pour lesquelles les deux électrodes indiquaient la même valeur à 2 mV près.

TABLEAU I

POTENTIELS D'ÉQUILIBRE ET POTENTIEL DE DEMI-RÉDUCTION A pH 7, 27°

Le facteur $\frac{RT/2f}{\log_{10} e}$ est égal, à 29.7 mV à 27°.

E *mV*	*pH*	*(D-lactate)*/*(Pyruvate)*	*E'*$_0$ *mV*	*Remarques*
—186	6.93	1	—190	Le potentiel des électrodes n'a atteint l'équilibre que lentement
—191	7.05	1	—188	
—189	6.93	1	—193	
—175	6.70	1	—193	
—172	6.70	1	—190	
—175	6.70	1	—193	
—202	7.17	2	—183	
—191	6.80	3.22	—188	
—188	6.74	3.22	—188	
			$\bar{m}$ = —188	
			σ = 3.2	
—195	7.17	1	—184	Le potentiel des électrodes atteint l'équilibre en 2 h et reste stable 20 h
—205	7.14	2	—188	
—202	7.17	2	—183	
—192	6.78	3.22	—190	
—191	6.73	3.22	—192	
			$\bar{m}$ = —190	
			σ = 3.2	
—184	6.73	1	—200	Equilibre atteint à partir des potentiels inférieurs
—176	6.81	1	—187	
			$\bar{m}$ = —194	
			σ = 6	

Nous avons calculé séparément (Tableau I) les résultats de ces deux groupes d'expériences: on voit qu'elles ont donné pour E'_0 des valeurs peu différentes soit —190.1 mV ($\sigma = 3.2$ mV) et —188.7 mV ($\sigma = 3.2$ mV). Nous retiendrons le chiffre —190 mV correspondant à la série des meilleurs déterminations.

TABLEAU II

COMPARAISON DES DIFFÉRENTES DÉTERMINATIONS DU POTENTIEL DE DEMI-RÉDUCTION À pH 7 DU SYSTÈME LACTATE–PYRUVATE

Potentiel en mV.

Températures	*15°*	*25°*	*27°*	*35°*	*37°*
Barron et Hastings[4]	—137*	—158*	—161*	—179	—183*
Wurmser et Mayer Reich[2]					—178
Banga *et al.*[3]					—181
Burton et Wilson[5]		—190			
Ce travail			—190		

* Valeurs calculées d'après les données de E^0 à 15 et à 35°[4].

CONCLUSION

La valeur du potentiel de demi-réduction E'_0 du système lactate–pyruvate à 27° et à pH 7 a été trouvée égale à -190 mV avec une erreur standard de 3.2 mV. Les valeurs que l'on en déduit sont pour E^0: $+226$ mV, et pour ΔF^0: $+10.4$ kcal.

Pour comparer ces résultats avec les anciennes déterminations, il faut tenir compte de la grande variation des valeurs de E'_0 avec la température; on peut calculer, d'après les données de BARRON ET HASTINGS[4] les valeurs de E'_0 aux différentes températures.

Le Tableau II montre que l'écart entre les récentes déterminations et la valeur généralement admise est très important. Comparées à une même température, ces valeurs diffèrent de 30 mV environ.

Le résultat obtenu est en excellent accord avec les calculs de BURTON ET WILSON[5]. La valeur de la variation d'énergie libre donnée par ces auteurs doit donc servir de base lorsqu'il s'agit d'évaluer l'énergie libre des réactions du métabolisme où est impliquée l'oxydation du lactate en pyruvate. Mais contrairement à la suggestion de ces auteurs, on ne peut rendre responsable la méthode électrométrique de l'inexactitude des données anciennes. L'équilibre y avait déjà été obtenu dans les deux sens et le présent travail apporte sur ce point une confirmation tout à fait satisfaisante. La divergence existant avec les anciennes déterminations du potentiel d'oxydo-réduction a pour origine la stéréospécificité de l'enzyme qui n'était pas prise en considération.

RÉSUMÉ

Les mesures de potentiel, en présence de riboflavine et de la D-lacticodéshydrogénase de la levure, ont donné pour l'équilibre D-lactate $\rightleftharpoons$ pyruvate, à pH 7 et à 27°, la valeur $E'_0 = -0.190$ V. Cette nouvelle détermination correspond bien à la valeur prévue par BURTON ET WILSON. Elle diffère notablement des anciennes déterminations directes dans lesquelles la stéréospécificité des enzymes n'était pas prise en considération.

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SOME ATYPICAL ALGAL CHROMOPROTEINS*

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SUMMARY

1. The biliproteins of two varieties of *Smithora* (*Porphyra*) *naiadum* were compared under identical conditions of extraction and chromatographic isolation. Fresh extracts of var. *australis* contain B-phycoerythrin, allophycocyanin and a purple complex having absorption maxima at 565 and 610 mμ. Upon standing, C-phycocyanin was released from this purple pigment fraction. Fresh extracts of var. *naiadum* contain B-phycoerythrin, allophycocyanin and C-phycocyanin. Upon standing, C-phycocyanin of the fresh var. *naiadum* extracts apparently becomes incorporated in a purple fraction resembling that in fresh extracts of var. *australis*.

2. The *in vivo* phycoerythrin of the three examined *Phormidium* species appeared to be C-phycoerythrin, absorption maximum approx. 565 mμ. This phycoerythrin was also obtained *in vitro* when extracted rapidly. On standing, extracts of these three algae yielded phycoerythrins having two absorption maxima (approx. 542 and 566 mμ). The formation of these artifact phycoerythrins appeared to be enzymically accelerated.

INTRODUCTION

The presence in red and blue-green algae of four spectrally distinct biliproteins has long been recognized. These are the R- and C- variants of phycoerythrin and phycocyanin, so designated by SVEDBERG AND KATSURAI[1] on the basis of their usual occurrence in the Rhodophyta or in the Cyanophyta. More recently attention has focused on unusual biliproteins which may occur as dominant or as minor components; *e.g.*, B-phycoerythrin[2,3], allophycocyanin[3-5], and biliprotein complexes[3,6].

The biliproteins of *Smithora* (*Porphyra*) *naiadum****, an aberrant member of the Bangiales, are of particular interest. DAWSON[8] distinguished between two varieties of this species found on the Pacific Coast of North America. Both are epiphytes on sea grasses; var. *naiadum*, the larger plant of the two, grows north of Point Conception, California, while var. *australis* is found south of this location. AIRTH AND BLINKS[2,3] have worked extensively on the biliproteins of the northern variety and have isolated in crystalline purity a new phycoerythrin which they designate B-phycoerythrin. That this pigment is also present *in vivo* is apparent from the thallus

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[***] HOLLENBERG[7] very recently placed this alga in the new genus *Smithora* because of its unusual method of reproduction.

absorption curves for the northern variety determined earlier by HAXO AND BLINKS[9]. B-phycoerythrin or a pigment with a very similar absorption curve also occurs in some other red algae; *i.e.*, *Porphyridium cruentum*[1,10], *Rhodosorus marina*[11], and *Rhodochorton floridulum*[12].

In this laboratory the biliproteins of the southern variety of *Smithora naiadum* were found to differ in some respects from those reported for the northern variety[13]. Through the courtesy of Professor L. R. BLINKS, one of us (C. Ó.) was afforded the opportunity of working at the Hopkins Marine Station, Pacific Grove and of comparing the biliproteins of the northern and southern varieties.

MATERIALS AND METHODS

Smithora naiadum var. *australis* (southern form) was collected at La Jolla, California, and var. *naiadum* (northern form) was obtained at Pacific Grove, California. Both were growing epiphytically on the sea grass *Phyllospadix*. The *Smithora* thalli were extracted overnight in distilled water at 3–5°. The extracts were centrifuged at 30,000 × *g* in the cold for at least 30 min, and the supernatant biliproteins were then separated by chromotography on tricalcium phosphate gel[4,14]. The column was developed with 1 % NaCl followed by phosphate buffer pH 6.0–6.5 of increasing concentrations.

The *Phormidium* spp. were cultured in sea water enriched with nutrient salts[15] and with trace elements[16]. The cultures were grown at 20–25° in a window receiving a northern exposure. We are indebted to Dr. L. PROVASOLI for supplying the culture of *P. persicinum*.

RESULTS

B-phycoerythrin

Four colored bands were obtained when the fresh aqueous extracts of *Smithora naiadum* var. *australis* (southern form) were chromatographed on tricalcium phosphate with 1 % NaCl. The leading pink band was eluted with 0.0025–0.01 *M* phosphate. Its principal absorption peak was at 542 mμ, while there was a smaller one at 560–562 mμ (Fig. 1); its spectrum corresponds with that of B-phycoerythrin. The second band contained a purple pigment which was eluted with 0.05 *M* phosphate. This purple pigment ($\lambda_{\max}$ 565 and 610 mμ) is somewhat reminiscent of R-phycocyanin ($\lambda_{\max}$ approx. 555 and 615 mμ) but differs from the latter in the shape of its absorption curve, as well as in its instability, which is discussed below. The third band had a diffuse pink color and was not studied further. The fourth band contained a strongly adsorbed blue pigment which was eluted with 1 *M* phosphate. This pigment was allophycocyanin, with an absorption maximum at approx. 650 mμ.

Immediate rechromatography of the second, purple fraction (Fig. 1) indicated it to be homogeneous and apparently stable; it again formed a single band and its absorption spectrum remained unaltered. However, solutions of the chromatographically purified purple pigment, which had been stored at 3–5° for a week, were found to be heterogeneous. Chromatograms of such aged solutions contained several poorly separated purple zones plus a leading blue eluate with an absorption maximum at 610 mμ and a slight "shoulder" at 565–575 mμ. The latter spectrum is suggestive of slightly impure C-phycocyanin.

In contrast to chromatograms of fresh extracts, those obtained with whole extracts that had aged for more than a week in the refrigerator presented a quite different picture. Under these conditions the leading zone was C-phycocyanin (maximum at 610 mμ) and this was followed by B-phycoerythrin and allophycocyanin.

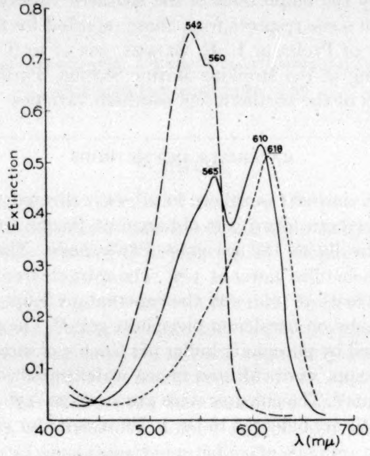

Fig. 1. Absorption spectra of *Smithora naiadum* biliproteins: –––––, B-phycoerythrin *ex S. naiadum* var. *australis*; ———, purple fraction *ex S. naiadum* var. *australis*, both in phosphate buffer pH 6.5; -------, C-phycocyanin *ex S. naiadum* var. *naiadum*, in dilute saline.

Three main bands were obtained when the fresh aqueous extract of *Smithora naiadum* var. *naiadum* (northern form) was similarly prepared and chromatographed. Here the first pigment to be eluted was not B-phycoerythrin but C-phycocyanin (absorption maximum at 618 mμ, Fig. 1). B-phycoerythrin and allophycocyanin were the other principal biliproteins obtained from this alga. The comparison of the fresh extracts thus revealed the presence of free C-phycocyanin in the northern but not in the southern form. In var. *australis*, C-phycocyanin apparently occurs in the freshly prepared extracts as a purple complex from which it is released when the extract is aged for a week or more. When the var. *naiadum* extract was aged for four days, C-phycocyanin no longer appeared on the chromatogram. B-phycoerythrin now was eluted first, followed by a purple eluate resembling the purple complex in fresh extracts of the southern form. The $\lambda_{\text{max.}}$ were similar (567 and 615 mμ), but the relative heights of the two peaks differed.

BLINKS[6] speculated that the purple pigment from var. *naiadum* might be a phycoerythrin–phycocyanin complex. AIRTH AND BLINKS[3] have recently discarded this idea and conclude that their data can be more readily interpreted by assuming that both B-phycoerythrin and C-phycocyanin exist in fresh extracts in two different forms, characterized by their electric charge and adsorptive properties respectively. However, the present chromatographic evidence suggests that in the var. *naiadum* examined by us, the "complex" is an artifact formed during storage of an aqueous extract, while the purple pigment of fresh var. *australis* extract may represent a native complex.

AIRTH AND BLINKS[2] found that B-phycoerythrin of var. *naiadum* was spectrally altered when the chromoprotein was recrystallized three times at pH 4.5, the shoulder at 565 mμ being lost. A similar spectral change was noted in the present study with the B-phycoerythrin of var. *naiadum* but not of var. *australis*. When the extract of var. *naiadum* was chromatographically fractionated by elution with acetate (pH 5.1) rather than phosphate buffer (pH 6.0), the spectrum of its B-phycoerythrin lacked a 565-mμ peak, but the spectrum of B-phycoerythrin form the southern form was unaltered.

C-phycoerythrin and artifacts

HAXO, ÓHEOCHA AND NORRIS[4] reported that *Phormidium ectocarpi* contained a two-peaked phycoerythrin (maxima at 544 mμ and 564 mμ). Reexamination has shown that the spectral characteristics of the phycoerythrin obtained from this and two other *Phormidium* species (*P. persicinum* and *P. fragile*) vary with the extraction method. When extracted for a few hours in distilled water, *P. ectocarpi* gave a brilliantly fluorescing single-peaked solution, having only one absorption maximum (565 mμ). When extraction was continued overnight, the absorption spectrum of the extract was now two-peaked, as previously reported[4]. This extract also fluoresced brilliantly and on chromatography yielded only one fraction, which was still double-peaked (the ratio of the extinctions at the maxima remained constant). Intact *P. ectocarpi* filaments possess only one pronounced absorption peak (at approx. 565 mμ). It would seem, therefore, that the native phycoerythrin of this alga is the C-variety.

The two peaked phycoerythrin, apparently an artifact not present *in vivo*, was also found to occur in extracts of *P. persicinum* and *P. fragile* under certain conditions. When examined earlier[4], *P. persicinum* appeared to contain only C-phyco erythrin (maximum at 560 mμ). Upon reexamination it was noted, however, that extracts which had been prepared by steeping filaments for 6-18 h in distilled water at 3° now exhibited two maxima at 542 and 568 mμ. Fractionation by chromatography or ammonium sulphate precipitation yielded a two-peaked phycoerythrin. A brownish-black, non-fluorescent dialysable substance which was also present gradually increased in amount when the extracts were stored in the cold. After about four days the phycoerythrin was replaced by such a brown pigment. The nature of this reaction has not been determined; however, it resembles melanization of proteins, an enzymically accelerated reaction[17, 18]. Extracts of other algae examined by us are generally stable under these conditions.

The C-variety also appears to be the main if not the only phycoerythrin of *P. fragile*. When held for a few hours at 0°, its extract appeared to contain a single-peaked phycoerythrin and a trace of phycocyanin. However, chromatography revealed a heterogeneity in the phycoerythrin. The first relatively faint zone from a 6-h extraction was double-peaked (545 and 567 mμ) and the remainder of the phycoerythrin was single-peaked (maximum at 563 mμ, Fig. 2). The relative amount of the two-peaked phycoerythrin increased when the extract was held for 36 h at 0°.

The original 6-h extract of *P. fragile* was precipitated with ammonium sulphate and kept in the dark at 0° for 3 weeks. This precipitate was dialysed against distilled water in the cold. Some of the pigment did not redissolve and was centrifuged off; the absorption spectrum of the fluorescing supernatant was two-peaked, as was the single red zone it formed on the column. On the other hand, the single-peaked C-phycoerythrin obtained by chromatography from 6-h extracts of *P. fragile* (Fig. 2) remained stable in phosphate buffer (pH 6.8; 0°) for days.

It appears, therefore, that the two-peaked phycoerythrin obtained from this and possibly the other two species of *Phormidium* does not exist *in vivo* but is formed in whole aqueous extracts by the action of enzyme(s) which can be removed by chromatography. The formation of the two-peaked phycoerythrin was not retarded by approx. 20 % ammonium sulphate.

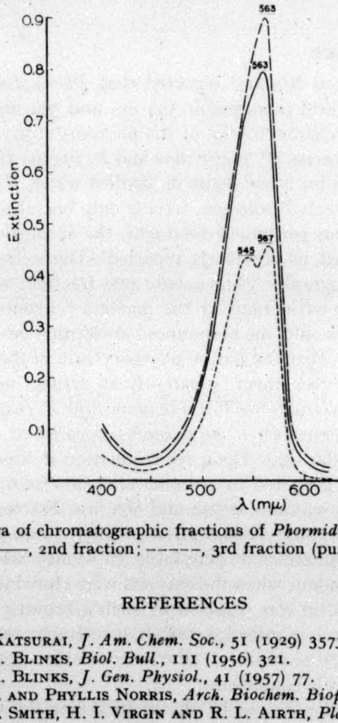

Fig. 2. Absorption spectra of chromatographic fractions of *Phormidium fragile* extracts: -------, 1st fraction; ———, 2nd fraction; – – – – –, 3rd fraction (pure C-phycoerythrin).

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Short Communications

A contribution to the problem of α-terminal groups of the polynucleotide chain of cytoplasmic ribonucleic acids*

On the basis of investigations of terminal groups of RNA, two principal types of the ribopolynucleotide chain were postulated to exist some time ago[1] (Fig. 1). RNA of the first type yields on alkaline hydrolysis one terminal nucleoside (the α-terminal group) and one terminal 3′,5′-diphosphonucleoside (the ω-terminal group) in addition to a mixture of (2′ + 3′)-nucleotides. On hydrolyzing the polynucleotide of the second type, only (2′ + 3′)-nucleotides are obtained. The α-terminal group of the chain can be determined in this case only after previous cleavage of the 3′-phosphate group of the α-terminal nucleotide by phosphomonoesterase; in the alkaline hydrolysate the corresponding nucleoside can then be found.

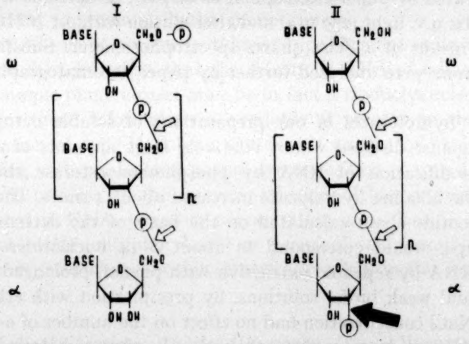

Fig. 1. The two principal types of the ribopolynucleotide chain (I, II). The white arrows indicate the cleavage of the 3′,5′-phosphodiester bridge on alkaline hydrolysis; the black arrow—the phosphate end group split off by phosphomonoesterase.

An exact determination of the α-terminal groups of polynucleotides of both types was considerably complicated by oligonucleotides, commonly accompanying RNA[2, 3]. These oligonucleotides could not be removed from the analyzed preparations by usual procedures, as *e.g.* precipitation, ultracentrifugation and dialysis against distilled water. It was found, however, that a marked improvement can be achieved in this respect by very simple means.

Cytoplasmic RNA was prepared from the soluble fraction of mouse-liver and pig-pancreas homogenates by phenol extraction[4]. Our preparations were purified by

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Abbreviations: RNA, ribonucleic acid.

* "α-terminal group" is applied to a nucleotide attached to the polynucleotide chain by a phosphodiester bond at the C′-5 atom; "Ω-terminal group" to a nucleotide attached at its C′-3 atom.

repeated extraction with phenol, several-fold precipitation with ethanol and by dialysis for 48–72 h against distilled water, using a cellophane sac (Kalle and Co.). In some experiments, RNA was further purified by dialysis for 48 h against 2 *M* NaCl[5,6]. Chlorides were removed by subsequent dialysis for 24 h against distilled water. All operations were carried out at 0–4°.

The modification of RNA by phosphomonoesterase, partially purified from human hypertrophic prostate glands, was achieved according to MARKHAM AND SMITH[7]. More than 90 % of total inorganic phosphate was split off during the first 6 h of incubation at 38°. Therefore the incubation was stopped after 6 h by adding an equal volume of water-saturated phenol and RNA prepared by phenol extraction and ethanol precipitation as described above. The modified RNA obtained was further purified either by dialysis against distilled water or against 2 *M* NaCl. A control experiment was performed parallelly in which the enzyme preparation was added immediately before mixing the incubation mixture with phenol.

RNA was hydrolyzed by 0.3 *N* KOH at 38° for 20–22 h. Hydrolysis was terminated by adding an equivalent amount of 3 *N* $HClO_4$ to the cooled solution; $KClO_4$ was centrifuged off, washed with 0.1 ml ice-cold distilled water and the combined supernatants were used without delay for further analyses. Nucleosides and nucleotides were separated by paper electrophoresis at pH 7.4, detected by photographing in monochromatic u.v. light (257 mμ) and after elution with 0.1 *N* HCl quantitatively determined by means of a Zeiss quartz spectrophotometer. Substances moving in the nucleoside zone were analyzed further by paper chromatography in *n*-butanol–water.

In alkaline hydrolysates of our preparations of soluble cytoplasmatic RNA, dialyzed only against distilled water, relatively large amounts of nucleosides were found. After modification of RNA by phosphomonoesterase the proportion of nucleosides in the alkaline hydrolyzate increased about 4 times. The average length of the polynucleotide chain calculated on the basis of the determined number of α-terminal groups, would correspond to about 8–14 nucleotides per chain. The purification of RNA by repeated extraction with phenol, prolonged dialysis against distilled water and weak buffer solutions, by precipitation with ethanol at various pH values and NaCl concentration had no effect on the number of α-terminal groups determined. A marked decrease was achieved only after a dialysis of RNA against 2 *M* NaCl. During this dialysis some 3–5 % of the material present in our RNA preparations passed through the cellophane membrane. In alkaline hydrolysates of thus purified preparations of RNA, 1 nucleoside for every 90–100 nucleotides was detected. After the modification by phosphomonoesterase the number of nucleosides increased only very slightly.

According to some sources in the literature the soluble liver RNA incorporates under optimal conditions one nucleotide remaining in the α-terminal position of the type I polynucleotide, for every 98 nucleotides[8]. A similar number of α-terminal groups was found in the soluble liver RNA by DUNN[9] by chemical analysis of alkaline hydrolyzates. Both findings are in good agreement with the results of our experiments. The molecular weight calculated from these results corresponds approximately to 30,000–35,000, which is in agreement with the values derived for this RNA fraction by methods of physical chemistry[4].

In the α-terminal groups of type I polynucleotide cytosine nucleotides were mostly detected together with a small amount of uracil nucleotides. Several reports in the literature indicated the exceptional position of α-terminal cytosine nucleotides and in a lesser degree also of those of uracil, in the incorporation of nucleotides from corresponding nucleoside triphosphates into RNA, which may be of importance in some intermediary reactions of protein synthesis[8,10,11]. The results of our experiments are in good agreement, both quantitatively and qualitatively with the findings made in laboratories where the above incorporation processes were investigated. Thus it appears that practically all α-terminal groups of type I polynucleotides—at least where soluble liver cytoplasmic RNA is concerned—can participate actively in incorporation processes.

The RNA preparations used here contained a certain amount of type II polynucleotides in addition to the bulk of type I polynucleotides. However, the increase in the percentage of nucleosides in hydrolysates following the modification of RNA by phosphomonoesterase was rather variable. When the preparations were dialyzed against a NaCl solution prior to hydrolysis, the increase in question was very slight even after a previous enzymic modification. There was at most 1 polynucleotide of type II for every polynucleotide of type I; in many preparations the type I polynucleotides predominated. However, the possibility of existence of type II polynucleotides with a 2′,3′-cyclic α-terminal phosphate group[7] cannot be excluded, this group being resistant to prostate phosphomonoesterase.

The biochemical role of polynucleotides of type II is not quite clear at present. Some findings indicate, however, that the infectious RNA of tobacco mosaic virus and some other simple plant viruses may be in fact a ribopolynucleotide of type II (see ref. 2, 3). Not much information is available at present on the mechanism of the actual formation of type II polynucleotides. It cannot be excluded at present that we are dealing with products of partial decomposition of type I ribopolynucleotides; even less so because all the known pathways of biosynthesis of compounds structurally related to RNA—whether polymeration of nucleoside diphosphates by bacterial polynucleotide phosphorylase[12] or terminal incorporation of nucleoside triphosphates mentioned above—result practically solely in the formation of polynucleotides of type I.

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Tryptophan content and tryptophyl sequences of proteins from six plant viruses

Previous studies on the selective cleavage of C-tryptophyl bonds in proteins with N-bromosuccinimide have revealed the presence of Try·Ala, Try·Lys and Try·Thr sequences in tobacco mosaic virus protein[1-3]. This communication contains data on the tryptophan content of, and the tryptophyl sequences occurring in, proteins from five strains of TMV—masked (M), Holmes rib grass (HR), green aucuba (GA), yellow aucuba (YA) and J14D1—and from cucumber virus 4 obtained by the application of the above technique. Such chemical data provide useful criteria for the classification of related viruses, and can indicate the extent, if any, of structural changes in limited parts of the protein molecule resulting from mutational events.

The protein samples were prepared from the respective viruses by a method used for the preparation of TMV protein[4]. The tryptophan content of the samples was calculated from their u.v. spectra in 0.1 N NaOH[5] and from the decrease in absorbancy at 280 mμ which results on oxidation with NBS[6,3]. The results are recorded in Table I.

TABLE I

TRYPTOPHAN CONTENT OF SIX VIRUS PROTEINS

Values in parentheses represent moles tryptophan/mole protein, assuming a common peptide-chain molecular weight of 18,000. Values in the last two columns are for the viruses and will need to be multiplied by a factor 100/94 to obtain values for the proteins. The colorimetric values reported in the literature are by the glyoxylic acid reaction.

Protein sample	*Tryptophan (%)*			
	Spectrophotometry in 0.1 N NaOH	*Based on NBS oxidation*	*Literature values*	
			Microbiological[15]	*Colorimetry*[12]
M	3.43 (3.03)	3.54 (3.12)	2.2	4.3
GA	3.39 (2.99)	3.41 (3.01)	2.1	4.2
YA	3.27 (2.95)	3.49 (3.07)	2.1	4.2
J14D1	2.99 (2.64)	3.71 (3.26)	2.2	4.4
HR	2.73 (2.41)	1.78 (1.56)	1.4	3.5
Cucumber virus 4	1.20 (1.05)	1.21 (1.06)	0.5	1.4

The amount of NBS consumed, per mole of tryptophan, to obtain minimum absorbancies at 280 mμ was as follows: M, 3.4; GA, 3.3; YA, 3.3; J14D1, 2.8; HR, 9.3. These values apply to oxidations performed in 70% acetic acid and represent the ratio of moles NBS consumed to moles tryptophan calculated to be present in the sample based on the minimum extinction at 280 mμ obtained on oxidation. The high value of 9.3 obtained for HR may be due to the comparative inaccessibility to the reagent of the tryptophan residues in this protein. The data in Table I shows that M, GA, YA and J14D1 contain three moles tryptophan per 18,000 g of protein, while Cucumber virus 4 has only one. This is in accord with the suggestion that while M, GA, YA, J14D1 and HR may be considered to be strains of TMV, cucumber virus 4 does not qualify for the same status[7]. The difference is strikingly borne out also by

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Abbreviations: NBS, N-bromosuccinimide; TMV, tobacco mosaic virus; M, HR, GA, YA, masked, Holmes rib grass, green aucuba, yellow acuba, respectively (strains of TMV).

the isolation of N-acetyl-Ser·Tyr from TMV, M, GA, YA, J14D1 and HR[8], while the N-terminal sequence of cucumber virus 4 was found to be N-acetyl-Ala·Tyr·AspNH$_2$·Pro·Ileu·Thr·Ser[9]. It is pertinent to point out further that the two methods of tryptophan estimation give rather concordant results, except for HR for which the NBS method gives a considerably lower value than the other method. Indeed, these values do not enable one to decide between the presence in HR of only two, or of three, residues of tryptophan per mole. In using NBS oxidation as a method of tryptophan determination it may be necessary to recognise the possibility of the presence of residues in certain proteins which may be resistant to oxidation either due to unfavourable steric factors in the original protein or because the protein in which some of the tryptophan residues have been fully oxidized may have changed in shape or structure in such a way that the rest of the residues become inaccessible to the reagent. The need for caution is illustrated by TMV protein which was reported to contain only two tryptophan residues per 18,000 g (see ref. 3) and later shown to contain three residues of the amino acid by the isolation of a peptide, from a tryptic hydrolysate of the protein, containing the third residue.

Separate samples of the proteins were oxidized with varying amounts of NBS and the new N-terminal groups that resulted were determined by the fluorodinitrobenzene method as used in a previous investigation[3]. The results are recorded in

TABLE II

YIELD OF N-TERMINAL RESIDUES ON NBS OXIDATION

Values are expressed as number of moles/mole of protein of weight 18,000. The entry "artefact" refers to a spot, in the Levy two-dimensional chromatogram of DNP-amino acids, occurring in the region of phenylalanine and whose identity has not been established.

Protein sample	*Conditions of reaction (28°, 1 h)*		
	70% AcOH, 3 moles NBS/mole tryptophan	*70% AcOH, 6 moles NBS/mole tryptophan*	*6 M urea-AcOH, pH 4, 3 moles NBS/mole tryptophan*
M	Lys 0.22 Ala 0.06 Thr 0.15	Lys 0.04 Ala trace Thr trace	Lys 0.06
GA	Lys 0.09 Ala 0.04 Thr 0.05	Nil	Lys 0.03 Thr 0.07 (Ser 0.03, Val 0.06, "Leu" 0.07, artefact 0.01)
YA	Lys 0.17 Ala 0.13 Thr 0.19 (Ser 0.01)	Lys 0.12 Ala 0.11 Thr 0.14 (Traces of Asp and artefact)	Lys 0.05 Ala 0.01 Thr 0.06
J14D1	Lys 0.08 Ala 0.10 Thr 0.03	Ala 0.04 Thr 0.04 (Trace artefact)	Lys 0.05 Ala 0.15
HR	Ala 0.08 Thr 0.06 (Artefact 0.12)	Ala 0.07 Thr 0.12 (Artefact 0.12)	Nil
Cucumber virus 4	Thr 0.06 (Ser 0.01)	Nil	

Table II. The best yields are obtained in 70 % acetic acid medium with the use of 3 moles NBS to every mole tryptophan present. The use of 6 *M* urea at pH 4 shows lack of cleavage of bonds, poor cleavage yields and in the case of GA even some non-specific fission of bonds. This is similar to earlier observations on cleavages with other proteins[3] although with the serum albumins urea does provide a good medium for cleavage[3, 10]. From the results recorded in Table II, it can be concluded that M, GA, YA and J14D1 share in common with TMV the sequences Try·Ala, Try·Lys and Try·Thr. HR contains, in common with TMV and the above, the sequences Try·Ala and Try·Thr while the third sequence, if there is one, has not been identified. Cucumber virus 4 seems to contain the Try·Thr sequence. It is a matter for speculation whether these sequences (of particular interest are M, GA, YA and J14D1) occur in the same structural environment in which they are found in TMV protein. In this connection, it may be recalled that TMV, M and YA are known to contain the same C-terminal hexapeptide sequence –Thr·Ser·Gly·Pro·Ala·Thr and HR contains instead –Thr(Thr,Ala)Pro·Ala·Thr[11]. In serological behaviour cucumber virus 4 and HR differ from the other four[12].

The M protein on tryptic hydrolysis, under conditions described for TMV protein[13], gave a 25 % yield of a peptide insoluble at pH 4.7, and with properties similar to the I-peptide from TMV protein[14] (RAMACHANDRAN AND TSUGITA, to be published). The lack of a reactive amino group in this peptide material, and the report of an acetylated N-terminus in the M protein[8], would indicate that this peptide comes from the N-terminal portion of the molecule. NBS oxidation revealed the presence of approximately a mole of tryptophan in 5000 g of this peptide and cleavage with 3 moles of NBS in 70 % acetic acid gave a yield of 9 % of 1 mole of alanine as a new N-terminal residue, thus indicating the presence of the Try·Ala sequence in the material. Since the Try·Ala sequence has been reported also to be present in the I-peptide from TMV protein[3], it may be concluded that the Try·Ala sequence in TMV and M proteins occurs in the same N-terminal portion of the protein molecule.

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Comparison of *dl*- and *d*-α-tocopherol in the reactivation of the DPNH-cytochrome *c* reductase of isooctane-treated heart-muscle preparation

NASON AND LEHMAN's[1] observations that the DPNH-cytochrome *c* reductase of particulate preparations is inactivated by shaking the suspension with isooctane, and reactivated by α-tocopherol, have been confirmed in several laboratories[2–5]. Recent studies[3–6] have, however, given further experimental support to the suggestion[2] that this phenomenon is not due to extraction of α-tocopherol by isooctane, as believed by NASON AND LEHMAN[1], but is caused by direct inhibition by isooctane. It seems likely that isooctane is adsorbed on, or dissolved in, the lipoprotein complex which constitutes the respiratory chain, and that α-tocopherol and a number of other lipids reverse the inhibition by dissolving and removing the isooctane.

However, there is one observation that appears difficult to fit into this picture of a physical mechanism of reactivation after isooctane inhibition. Both DEUL *et al.*[2] and WEBER *et al.*[4] have reported that *d*-tocopherol was more effective than *dl*-tocopherol. Since it would not be expected that the lipid solubility of tocopherol would be affected by its stereochemical configuration, this question has been further examined, using pure samples of *d*- and *dl*-α-tocopherol obtained from different sources.

Two preparations of *dl*-α-tocopherol were used: (i) a commercial preparation of Hoffmann-La Roche and Co.; (ii) a specially purified preparation kindly supplied by Dr. D. HEPDING of Merck A.G., Darmstadt. Two preparations of *d*-α-tocopherol were also used: (i) a commercial preparation of Mann Research Laboratories; (ii) a purified preparation kindly supplied by Dr. G. BAXTER of Distillation Products Industries. Tocopherol suspensions (1 mg/ml) were made by adding 1.7 volumes 0.2 % serum albumin in 0.1 *M* Sørensen phosphate buffer, pH 7.4, to 0.3 volume of a 0.67 % ethanolic solution of tocopherol. At this concentration the tocopherol remained in suspension and the tocopherol content of the emulsion as measured by the method of EMMERIE AND ENGEL[7] agreed closely with the expected concentration.

The enzyme preparation was a KEILIN AND HARTREE heart-muscle preparation made from horse heart[8]. A concentrated suspension (10–40 mg protein/ml) was treated with isooctane as described by DEUL *et al.*[2]. The DPNH-cytochrome *c* reductase was measured according to DEUL *et al.*[2] with a final concentration of heart-muscle preparation of between 0.004 and 0.04 mg/ml.

The two *dl*-α-tocopherol samples were indistinguishable so far as the effects on the untreated and isooctane-treated heart-muscle preparation were concerned. The same was true of the two *d*-α-tocopherol samples. The data on the relative activity of *dl*- and *d*-α-tocopherol in restoring DPNH-cytochrome *c* reductase activity to isooctane-treated preparations are presented in Table I. It is apparent that there is no difference in the reactivation capacity of the *dl*- and *d*-forms of α-tocopherol. No satisfactory explanation can be offered for the difference between the results shown in Table I, and those of previous studies[2,4].

Three sets of experiments provided additional evidence that the inactivation brought about by shaking the preparation with isooctane is due to inhibition by isooctane, rather than to extraction by the isooctane of a component of the enzyme system.

Abbreviation used: DPNH, reduced diphosphopyridine nucleotide.

TABLE I

COMPARISON OF *d*- AND *dl*-*α*-TOCOPHEROL IN RESTORING DPNH-CYTOCHROME *c* REDUCTASE ACTIVITY TO ISOOCTANE-TREATED HEART-MUSCLE PREPARATION

The specific activity of the untreated preparation, which varied between 0.07 and 0.21 μmole DPNH/mg protein/min, is put equal to 100 in each experiment; all other activities are expressed as % of values for untreated preparation. The same concentration of heart-muscle preparation was used in the measurement of the activities of treated und untreated preparations. Albumin in the amounts supplied by the tocopherol emulsion had no effect on the isooctane-treated preparation.

Expt.	*Untreated preparation*			*Isooctane-treated preparation**			*Final concn. tocopherol (μM)*
	No addition	+ dl-*tocopherol*	+ d-*tocopherol*	*No addition*	+ dl-*tocopherol*	+ d-*tocopherol*	
1	100			18	44	40	7.7
		116	110		64	67	23
2	100	121		3	46	47	100
3	100		123	13	20	21	7.7
					41	39	15.4
		132			67	67	23
4	100	163		8	63	65	23
5	100			15	55	55	23
		200			130	130	46
					171	172	100
6	100			6	71	58	23
7	100		174	5	51	51	23
					69	68	31

* Shaking with an equal volume isooctane for 2 min at 0°. The isooctane layer was removed by centrifugation at 500 × *g* for 10 min. Additional isooctane was added and the shaking and centrifugation repeated 1–8 times (the number of isooctane treatments required to decrease the activity to below 20 % of the control varied greatly from preparation to preparation).

1. In experiments in which the enzymic activity was not decreased below 25 % of the control by a single isooctane treatment, marked inhibition could be unmasked by employing higher concentrations of the preparations in the measurement of the activity (Table II). With untreated preparation, the use of twice the concentration of enzyme produced a 2-fold increase in activity (Expts. 11 and 12), whereas progressive inhibition was found with increasing concentrations of the treated preparation. Also at these high enzyme concentrations, there was no difference between the ability of the two stereoisomers of α-tocopherol to reactivate the isooctane-treated preparation. The greater inhibition observed with more concentrated enzyme is presumably due to the presence of increasing concentrations of isooctane in the reaction mixture.

2. In two experiments, aliquots of isooctane-treated preparations with activities of 9 and 28 % of the control were lyophilized and reconstituted in distilled water. The reconstituted preparations had the enzymic activity restored to the extent of 90 and 92 %, respectively. This confirms the report of POLLARD AND BIERI[4].

3. The addition of isooctane to the reaction mixture in the cuvettes, prior to the addition of the enzyme preparation, completely blocked the DPNH-cytochrome *c* reductase activity.

The activation by α-tocopherol of the DPNH-cytochrome *c* reductase of untreated preparations (see Tables I and II) remains to be explained. Among possible explanations to be considered for this action of α-tocopherol is its ability to remove an endogenous inhibitor, or to increase the accessibility of added cytochrome *c* to the enzyme system on the particle in the same way as bile salts[9].

TABLE II

THE EFFECT OF CONCENTRATION OF UNTREATED AND ISOOCTANE-TREATED HEART-MUSCLE PREPARATION ON DPNH-CYTOCHROME *c* REDUCTASE AND THE EFFECT OF α-TOCOPHEROL

The specific activity measured at the most dilute concentration used is set at 100. Other activities are expressed as % of this value.

		Expt.						
		8	*9*	*10*	*11*		*12*	
Untreated preparation, diluted	1:8000		100	100	100		100	
+ *dl*-tocopherol					135		139	
+ *d*-tocopherol					119			
	1:4000	100			96		100	
Isooctane-treated* preparation, diluted	1:8000		103	89	100		25	
+ *dl*-tocopherol					114		66	
+ *d*-tocopherol					110		68	
	1:4000	40	67	52	58		8	
+ *dl*-tocopherol					126		22	
+ *d*-tocopherol					126		24	
	1:2000	16	18	19	12		4	
+ *dl*-tocopherol					54		4	13
+ *d*-tocopherol					56		4	14
	1:1000	5	11	10	5			
+ *dl*-tocopherol					11	24		
+ *d*-tocopherol					11	19		
Final concn. of tocopherol (μM)					23	46	7.7	46

* Treated once as described in Table I.

The observations reported in this paper are in agreement with the postulate that the reactivation of isooctane-treated preparations is essentially a physical phenomenon.

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β-Hydroxypropionaldehyde, an intermediate in the formation of 1,3-propanediol by *Aerobacter aerogenes*

A number of micro-organisms produce 1,3-propanediol during the course of glycerol fermentation[1]. The detection of acrolein[3] during glycerol fermentation led to the suggestion that acrolein is a precursor of 1,3-propanediol. However, later work[3] failed to confirm this. VOISENET[4] proposed β-hydroxypropionaldehyde as an intermediate. The identification of this aldehyde was based upon a color test which has not been demonstrated to be specific for β-hydroxypropionaldehyde. Recently this aldehyde has been postulated as an intermediate in the formation of 1,3-propanediol by *Lactobacillus*[5]. No unequivocal evidence for the occurrence of β-hydroxypropionaldehyde has as yet been presented.

We have investigated the pathway of this reaction with *Aerobacter aerogenes* (ATCC 8724) grown anerobically on glycerol. Suspensions of these organisms in 0.1 M potassium phosphate buffer, pH 7.4, containing 3 % glycerol convert from 35 % to 50 % of the glycerol to 1,3-propanediol. When this reaction is carried out in the presence of 0.6 M semicarbazide a carbonyl compound accumulates and no propanediol can be detected. Addition of an ice-cold 0.1 % solution of 2,4-dinitrophenylhydrazine in 2 N HCl to the cold cell-free reaction mixture yields a 2,4-dinitrophenylhydrazone which has an R_F in methanol-heptane[6] identical with that of the 2,4-dinitrophenylhydrazone derived from synthetically prepared β-hydroxypropionaldehyde[7]. The elementary analysis also corresponds to that of β-hydroxypropionaldehyde 2,4-dinitrophenylhydrazone. When the hydrazone formation is carried out at room temperature the 2,4-dinitrophenylhydrazone of acrolein is obtained, in accordance with previous reports[2]. Synthetic β-hydroxypropionaldehyde also yields the acrolein derivative under these conditions.

We therefore concluded that the material which is trapped when *Aerobacter aerogenes* acts on glycerol in the presence of semicarbazide is β-hydroxypropionaldehyde. In the absence of semicarbazide no β-hydroxypropionaldehyde accumulates and 1,3-propanediol is formed, β-hydroxypropionaldehyde thus fullfills the requirements of a metabolic intermediate in the conversion of glycerol to 1,3-propanediol.

As shown in Fig. 1, extracts of *Aerobacter aerogenes* catalyze the oxidation of DPNH by β-hydroxypropionaldehyde. This reaction proceeds much more rapidly with β-hydroxypropionaldehyde than with any other aldehyde tested. These findings suggest the following reaction sequence for the conversion of glycerol to 1,3-propanediol:

$$CH_2OHCHOHCH_2OH \rightarrow CH_2OHCH_2CHO \xrightarrow{DPNH} CH_2OHCH_2CH_2OH$$

β-hydroxypropionaldehyde thus serves as one of the hydrogen acceptors during the fermentation of glycerol. This ability to convert glycerol to a hydrogen acceptor seems to be essential for the fermentation of glycerol. Organisms which do not form 1,3-propanediol, and therefore, presumably cannot form β-hydroxypropionaldehyde, require the addition of hydrogen acceptors in order to ferment glycerol[1].

In addition to converting glycerol to β-hydroxypropionaldehyde, *Aerobacter aerogenes* is also able to convert other diols to the corresponding deoxyaldehyde. Ethyleneglycol is converted to acetaldehyde and 1,2-propanediol to propionaldehyde.

Abbreviation: DPNH, reduced diphosphopyridine nucleotide.

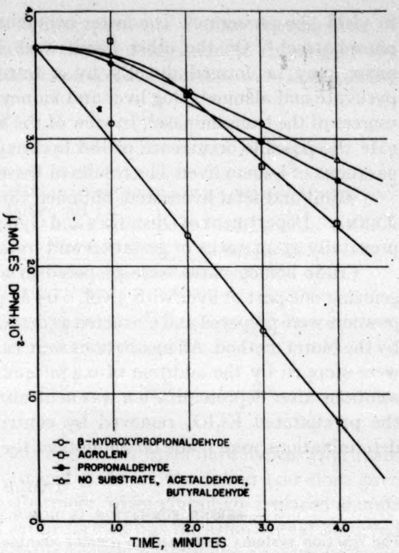

Fig. 1. The reaction mixture contained 3.7 µmoles DPNH, 20 µmoles substrate, 100 µmoles potassium phosphate buffer pH 7.0, 750 µg lyophilized bacterial extract. Total volume, 2.9 ml. Extract was prepared by grinding 2 g of lyophilized cells with 40 g alumina and extracting with 50 ml 0.01 *M* tris(hydroxymethyl)aminomethane buffer, pH 8.0. Extract was then treated with 1 g Darco G-60.

A number of diols containing four or more carbon atoms, as well as compounds containing amino and thiol groups were also incubated with *Aerobacter aerogenes*. These compounds were not converted to the corresponding deoxyaldehydes.

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Serine biosynthesis in human liver preparations

Two different transaminases have been shown to give rise to serine in mammalian systems. It has been demonstrated that one route for serine biosynthesis in the rat is by a transamination reaction between phosphohydroxypyruvate and glutamate

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Abbreviation: DPNH, reduced diphosphopyridine nucleotide.

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to yield phosphoserine[1]. The latter compound is hydrolyzed to serine by a specific phosphatase[2,3]. On the other hand, work in this laboratory has established that serine may be formed directly by a transamination reaction between hydroxypyruvate and alanine[4]. Dog liver and kidney and rabbit liver were shown to be good sources of the transaminase[5]. In view of the above results it was of interest to investigate the possible occurrence of the hydroxypyruvate–alanine transaminase in preparations of human liver. The results of these studies are presented here.

Adult and fetal livers were obtained through the help and courtesy of Dr. A. L. KENNAN, Department of Obstetrics and Gynecology. Fresh, normal liver was obtained prenatally at 40 weeks of gestation and from an adult during surgery.

Crude homogenates were prepared in a Potter–Elvehjem apparatus by homogenizing one part of liver with 3 vol. 0.05 *M* phosphate buffer, pH 7.4, at 0°. Acetone powders were prepared and extracted as described previously[4]. Protein was determined by the biuret method. All incubations were carried out at 37° for 75 min. The reactions were stopped by the addition of 0.4 ml 4 *N* $HClO_4$. The $HClO_4$ in the supernatant solutions after deproteinization was neutralized to pH 2 with 4 *N* KOH, chilled, and the precipitated $KClO_4$ removed by centrifugation. Serine and hydroxypyruvate determinations were made on aliquots of the resulting supernatant solutions.

TABLE I

SERINE FORMATION IN HOMOGENATES OF HUMAN LIVER

The reaction systems contained 50 µmoles alanine, hydroxypyruvate as indicated, 2 ml of 1:3 homogenate in a final volume of 4.5 ml, 0.05 *M* potassium phosphate buffer, pH 7.4.

Substrates	*Hydroxypyruvate*		*Serine formed µmoles*
	Added µmoles	*Remaining µmoles*	
Adult:			
Hydroxypyruvate	48.8	18.8	1.5
Hydroxypyruvate + Alanine	48.8	16.2	7.3
Fetal:			
Hydroxypyruvate	44.2	18.0	0.9
Hydroxypyruvate + Alanine	44.2	14.7	6.3
Non-enzymic:			
Hydroxypyruvate	47.8	46.4	0
Hydroxypyruvate + Alanine	—	—	0

For the determination of serine an aliquot containing from 2–10 µmoles of the amino acid was placed on a 1 × 6 cm column of Dowex 50, Na^+ form. The column was washed with 4 ml water and then eluted with 0.3 *M* sodium acetate buffer, pH 5.0. The serine fraction (2 to 8 ml of effluent volume) was collected and analyzed colorimetrically[6].

Crystalline lithium hydroxypyruvate was prepared according to the method of DICKENS AND WILLIAMSON[7] and assayed spectrophotometrically with DPNH and plant hydroxypyruvate reductase[8].

The results of the experiments with the homogenates are presented in Table I. With either the adult or the fetal liver preparations, significant serine formation was observed only in the incubation systems containing both hydroxypyruvate and alanine. These results indicate that the hydroxypyruvate–alanine transaminase is present in human liver. In order to confirm these findings and to investigate the specificity of the amino group donor, extracts of acetone powders were investigated (Table II).

TABLE II

SERINE FORMATION BY EXTRACTS OF ACETONE POWDER OF HUMAN FETAL LIVER

The reaction systems contained 50 µmoles of the amino acid, hydroxypyruvate as indicated, 1 ml of enzyme dialyzed overnight (23.3 mg protein) in a final volume of 2.5 ml, 0.02 *M* potassium phosphate buffer, pH 7.4.

Substrates	*Hydroxypyruvate*		*Serine formed µmoles*
	Added µmoles	*Remaining µmoles*	
Hydroxypyruvate	50.6	40.6	0.2
Alanine	50.6	—	0.2
Hydroxypyruvate + Alanine	50.6	34.6	5.6
Glutamate	50.6	—	0.2
Hydroxypyruvate + Glutamate	50.6	40.6	0.2

It can be seen that serine synthesis was dependent upon both hydroxypyruvate and alanine as substrates. Neither the keto acid alone nor the amino acid alone gave rise to a significant amount of serine. Furthermore, when glutamate replaced alanine as the amino group donor, the amount of serine formed was the same as that observed with hydroxypyruvate alone. This precludes formation of serine by another pathway utilizing glutamate as the amino group donor. An enzyme-product curve with the acetone-powder extract showed that the transamination, under the experimental conditions used, is a zero-order reaction. The above results clearly demonstrate that human liver contains the hydroxypyruvate–alanine transaminase.

The role of this transaminase in serine biosynthesis must await the demonstration of the metabolic origin of hydroxypyruvate. It is of interest to note that hydroxypyruvate is actively metabolized by these enzyme preparations as shown by the figures in Tables I and II. Preliminary studies with ^{14}C-labeled hydroxypyruvates indicate that a variety of products are formed from this compound.

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On the mechanism of N-acetylmannosamine formation

The enzymic formation of an aminohexose from uridine diphosphate-N-acetylglucosamine by an enzyme from rat liver was described by Cardini and Leloir[1]. The amino sugar formed by this enzyme was identified as N-acetylmannosamine by Comb and Roseman[2]. We wish to report results obtained when this reaction is carried out in tritium-enriched water (HTO).

The enzyme was prepared by the method of Cardini and Leloir[1], except that the liver extract was centrifuged at 100,000 × *g* for 1 h before $(NH_4)_2SO_4$ fractionation. Because of its instability, the enzyme was used immediately after preparation.

Radioactivity was determined with a proportional windowless gas-flow counter (Nuclear-Chicago Corporation). Approx. 0.3 μmole of sugar was plated on an area of 4.5 cm^2 for counting. The efficiency for tritium counting under these conditions is approximately 5 %, and the reproducibility about 10 %.

The reaction mixtures contained 50 μmoles tris(hydroxymethyl)aminomethane buffer, 20 μmoles cysteine, 20 μmoles $MgCl_2$, 0.6 ml enzyme (activity = 30 μmoles N-acetylmannosamine formed/h/ml), 10 μmoles uridine diphosphate-N-acetyl-glucosamine and 12.5 mC of HTO in a final volume of 2.6 ml, pH 7.8. The samples were incubated for 1 h at 37°, and the reaction stopped by heating at 100° for 90 sec. All the uridine diphosphate-N-acetylglucosamine had been converted to N-acetylmannosamine at the end of the incubation.

The water was removed by lyophilization. The residue was suspended in 6 ml water and the denatured protein removed by centrifugation. The supernatant fluid was deionized by passage through a 1 × 12 cm column of Amberlite MB-3, and the eluate containing the N-acetylmannosamine concentrated under reduced pressure. The N-acetylmannosamine was dissolved in two 20-ml portions of water, and each time was concentrated under reduced pressure. The sugar was finally isolated by paper chromatography using butanol–pyridine–water (6:4:3) (see ref. 3) as the solvent. In some experiments borate-treated paper was used[1]. The results were the same in both cases.

The results of a typical experiment are shown in Table I. The N-acetylmannosamine formed from uridine diphosphate-N-acetylglucosamine contained approx. 1 μatom tritium/μmole. When the enzyme was incubated with N-acetylmannosamine and the sugar isolated as described, it contained no radioactivity.

To ascertain the position of the tritium in the sugar molecule the N-acetylmannosamine was degraded as follows:

The N-acetylmannosamine was hydrolyzed for 3 h in 2 *N* HCl, and the mannos-

TABLE I

INCORPORATION OF TRITIUM INYO N-ACETYLMANNOSAMINE

Substrate	*Tritium in isolated N-acetylmannosamine counts/min/μmole*
Uridine diphosphate-N-acetylglucosamine	4120
Uridine diphosphate-N-acetylglucosamine	4370
N-acetylmannosamine — 7.5 μmoles	20

amine formed isolated by ion-exchange chromatography on Dowex-50(H$^+$) (see ref. 4). The mannosamine was degraded to arabinose with ninhydrin[5], and the arabinose isolated by paper chromatography using butanol–pyridine–water as the solvent. The arabinose was converted to arabonic acid by hypoiodite oxidation and the arabonic acid isolated by paper chromatography.

The results are shown in Table II and indicate that the tritium atom is attached to carbon atom 2 of the N-acetylmannosamine.

TABLE II

DEGRADATION OF N-ACETYLMANNOSAMINE

Compound	*Tritium counts/min/μmole*
N-acetylmannosamine	2100
Mannosamine	2370
Arabinose	2050
Arabonic acid*	5

* The arabinose was diluted to a specific activity of 450 counts/min/μmole before oxidation.

To determine whether the enzyme would incorporate tritium from water into uridine diphosphate-N-acetylglucosamine, the enzyme was incubated with uridine diphosphate-N-acetylglucosamine as described, but the reaction was stopped by heating to 100° after 40 % of the substrate had been converted to N-acetylmannosamine. The unreacted uridine diphosphate-N-acetylglucosamine was isolated by chromatography on Dowex-1 (formate)[6].

After lyophilization, the nucleotide was subjected to mild acid hydrolysis (pH 2, 100°, 20 min) and deionized by passage through an Amberlite MB-3 column. The radioactivity of the N-acetylglucosamine in the eluate was determined. In several experiments with different enzyme preparations no tritium was found in the N-acetylglucosamine.

No simple mechanism can be written for this reaction on the basis of these results; they indicate, however, that the mechanism differs from the previously studied uridine diphosphate glucose-4′-epimerase, which probably involves an oxidation–reduction, with diphosphopyridine nucleotide acting as a hydrogen acceptor[7,8].

Since no tritium was found in the uridine diphosphate-N-acetylglucosamine, the results would also exclude an oxidation–reduction mechanism in which the hydrogen acceptor was a compound like riboflavin which would exchange hydrogen with the medium[9].

Since all attempts to demonstrate uridine diphosphate-N-acetylmannosamine as an intermediate in this reaction have been negative[1,10], and since the enzyme will not incorporate tritium into uridine diphosphate-N-acetylglucosamine, or into N-acetylmannosamine, it is tempting to speculate that both the epimerization and the cleavage are catalyzed by the same enzyme.

The details of this interesting reaction probably can only be investigated with purer enzyme preparations. The enzyme, however, is very unstable, and attempts to purify it further have not met with much success[1,10].

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Paper chromatography of some guanidine derivatives

For the qualitative analysis of mixtures of amino acids, the use of paper chromatography, introduced by CONSDEN *et al.*[1] is unrivalled. This coupled with paper electrophoresis, utilized first by KONIG AND VON KLOBUSITZKY[2] and TISELIUS[3], makes the separation more complete. Thus, LISSITZKY, GARCIA AND ROCHE[4] characterized a few guanidine derivatives of biological interest including citrulline by employing chromatoelectrophoresis. However, their list does not include those guanidine derivatives which have since been isolated from animal origin. Therefore, a comparative chromatographic study of these newly isolated guanidine derivatives with respect to their behaviour towards different solvent systems was thought of some interest and utility.

Materials

Arginine monohydrochloride, creatine and glycocyamine were preparations of B.D.H., Poole, England.

Guanidinoethanol was prepared from ethanolamine and S-methylisothiourea in ammoniacal medium according to SCHUTTE[5], as described by VON THOAI AND ROBIN[6].

Guanidinoethanol phosphate was prepared according to PANT AND DUBEY[7] and purified by crystallizing several times with distilled water and methanol.

Taurocyamine was prepared by the method described by MORRISON, ENNOR AND GRIFFITHS[8] and purified by repeated crystallization to constant melting point (262–264°).

Lombricine was isolated from *Pheretima posthuma* by employing ion-exchange resins as described by PANT[9].

Analar chemicals were employed for all solvents.

Chromatography

Paper partition chromatography of CONSDEN *et al.*[1] was used. Both the two-dimensional technique of Datta, Dent and Harris[10] and the one-dimensional ascending method were employed.

A 250 mg % (w/v) solution of the guanidine derivatives was prepared and small volumes (20–25 μl) were applied on Whatman No. 1 filter paper sheets of suitable size and dried. The operation was conducted in glass aquarium tanks fitted with air-tight lids. The one-dimensional chromatograms (30 cm × 20 cm) were allowed to run at room temperature (31°) in the respective solvents for about 14 h in case of solvents I–IV and for 6 h in solvent V to a distance of about 25 cm.

TABLE I

R_F OF GUANIDINE DERIVATIVES DEVELOPED WITH DIFFERENT SOLVENT SYSTEMS

The proportions of the components of the solvent systems are by volumes. I, *n*-butanol–acetic acid–water (40:10:50); II, pyridine–isoamyl alcohol–water (80:40:70); III, pyridine–isoamyl alcohol–water–20% (v/v) aq. NH_3, sp. gr. 0.88 (80:40:40:10); IV, phenol (80%, w/v)–NH_3 (see text); V, methanol–water–acetic acid (80:20:10).

Solvent	*Arginine*	*Creatine*	*Guanidino ethanol*	*Guanidino ethylphosphate*	*Glycocyamine*	*Lombricine*	*Taurocyamine*
I	0.15	0.33	0.35	0.17	0.36	0.07	0.24
II	0.44	0.51	0.50	0.33	0.43	0.21	0.63
III	0.37	0.44	0.65	0.29	0.40	0.18	0.54
IV	0.76	0.82	0.82	0.49	0.61	0.48	0'57
V	0.68	0.72	0.72	0.66	0.70	0.36	0.56

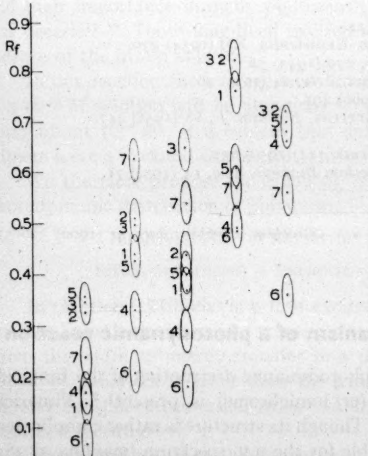

Fig. 1. Solvents same as for Table I. 1, arginine; 2, creatine; 3, guanidinoethanol; 4, guanidinoethyl phosphate; 5, glycocyamine; 6, lombricine; 7, taurocyamine.

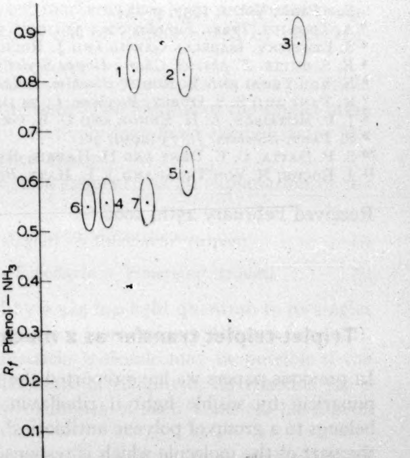

Fig. 2. Position of guanidine derivatives in two dimensional chromatograms. Numbers same as for Fig. 1.

The two-dimensional chromatograms (20 cm × 20 cm) were developed first with 80 % (w/v) aq. phenol for 6 h in an atmosphere of NH_3 provided by keeping a beaker containing aq. NH_3 (sp. gr. 0.88). The second development was with *n*-butanol–acetic acid–water (40:10:50, v/v/v) for 4 h. This solvent was prepared by mixing the ingredients thoroughly in a separating funnel and discarding the lower immiscible aqueous portion. After complete evaporation of the solvents from the chromatograms they were uniformly sprayed with the Sakaguchi reagents as described by PANT[9]. Appearance of pink spots characterized the position of the guanidine derivatives. Creatine was spotted by the method described by ROCHE *et al.*[11]. This consisted in spraying the chromatogram with a freshly prepared solution containing 0.1 ml diacetyl (added just before spraying) in 15 ml 1 % (w/v) α-naphthol in 6 % (w/v) NaOH. The appearance of a purple spot characterized creatine.

Table I shows the R_F's of different guanidine derivatives in the five solvent systems employed. Fig. 1 shows their comparative movements in the various solvents while Fig. 2 shows their position in two-dimensional chromatograms when 80 % phenol–NH_3 and *n*-butanol–acetic acid–water are employed as irrigating solvents.

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Triplet-triplet transfer as a mechanism of a photodynamic reaction

In previous papers we have reported the photodynamic destruction of the fungicide pimaricin by visible light if riboflavin (or lumichrome) is present[1,2]. Pimaricin belongs to a group of polyene antibiotics[3]. Though its structure is rather complicated, the part of the molecule which is responsible for the u.v. spectrum (maxima at 281, 292, 304 and 319 mμ) is practically limited to a tetraen-grouping. On irradiation in the presence of riboflavin with light of a wavelength of 445 mμ, the peaks at 304 and 319 mμ disappear rapidly, though pimaricin itself cannot absorb these quanta. So the light must be absorbed by riboflavin, which has maxima at 223, 268, 373, 445 mμ, and then transferred to pimaricin.

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Compounds with a tetraenstructure are usually very sensitive to free radicals and we originally supposed, therefore, that the photodynamic destruction was due to free radicals, which could have their origin in the decomposition of riboflavin by light[1].

From further work on this subject we have to conclude that neither an oxidation, as described in the literature on other photodynamic destructions[4,5] in which riboflavin is involved, nor a free-radical mechanism[6-8] is responsible for our reaction.

Though the structure of pimaricin is very complex we have nevertheless continued to use this compound, for a change in this molecule under influence of light may be easily detected spectrophotometrically. Moreover simple tetraens are rare or very labile compounds, whereas pimaricin is rather stable and commercially available*.

In this paper arguments will be given that a direct transfer of excitation energy from riboflavin or lumichrome to pimaricin by collision may be involved. It is not likely that this transfer proceeds via a riboflavin–pimaricin or lumichrome–pimaricin complex because we were not able to detect these complexes by absorption and fluorescence measurements.

SZENT-GYÖRGYI[9,10] has recently given some examples of charge-transfer complexes, but the concentrations used in his experiments ($10^{-3}\,M$) were much higher than those in ours ($< 10^{-5}\,M$). However, we cannot completely exclude intermediate complex formation.

Further observations of the reaction mentioned have brought us to the conclusion that the sensitizers riboflavin and lumichrome react in their triplet states. These triplet states have a rather long lifetime compared with the corresponding singlets and their importance in many photosensitized reactions has been recognized in the last decade[11,12]. These long-lived excited states can be demonstrated by phosphorescence of the frozen solutions.

In our reaction the average time for collision between riboflavin and pimaricin in a $10^{-5}\,M$ solution will be about 10^{-4} - 10^{-5} sec[13]. The lifetime of the singlet state being about 10^{-8} sec, it is evident that only relatively long-lived excited states like triplets have a reasonal chance to transfer their energy.

We therefore propose the following reaction sequence as an explanation of the photodynamic destruction of pimaricin:

$$\text{Riboflavin} + h\nu \longrightarrow \text{Riboflavin}^* \text{ (singlet)} \longrightarrow \text{Riboflavin}^* \text{ (triplet)} \qquad (1)$$

$$\text{Riboflavin}^* \text{ (triplet)} + \text{Pimaricin} \longrightarrow \text{Riboflavin} + \text{Pimaricin}^* \text{ (triplet)} \qquad (2)$$

In reaction (1) riboflavin is first excited by a 445 mμ light quantum to its singlet state and next a singlet-triplet transition takes place (conversion of the spin moment). From this state an energy transfer to a pimaricin molecule may be possible if the triplet of pimaricin is lower than the similar state of riboflavin, for reaction (2) is allowed by spin-conservation rules[14]. A few examples of such transfers have been described recently[12,15].

The overall result of this triplet-triplet transfer would be an excited pimaricin molecule. It is conceivable that a very sensitive compound like pimaricin with its reactive tetraen structure decomposes if it is excited to its triplet state. The lability of pimaricin is demonstrated by its sensitivity to u.v. light.

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* We wish to express our gratitude to the Royal Dutch Fermentation Industries, Delft, Netherlands, for supplying this fungicide.

The reaction mechanism first mentioned is supported by the effect shown by several substances upon the system riboflavin–pimaricin. A compound that quenches the phosphorescence of riboflavin is supposed to act in this way by facilitating the radiationless dissipation of excitation energy by collisions with molecules of the solvent. Consequently the lifetime of the triplet state is shorter. This means the triplet–triplet transfer is less probable.

It appeared that the quenching effect on the phosphorescence of the compounds tested ran completely parallel to the degree of inhibition of the photodynamic destruction of pimaricin in the combination riboflavin–pimaricin.

On the other hand, compounds without visually observable quenching showed no inhibition, but in many cases even accelerated the pimaricin destruction.

For illumination a Kromayer high-pressure mercury lamp was used. The aqueous solutions containing $2 \cdot 10^{-5}\,M$ riboflavin and $9 \cdot 10^{-6}\,M$ riboflavin were buffered with a 0.06 M phosphate buffer at pH 6.8. The distance between lamp and solution was 3 cm. By using an interference filter, light with a wavelength of about 443 mμ was obtained. The destruction of pimaricin was traced by measuring the extinctions at 304 and 319 mμ by means of a Unicam Spectrophotometer S.P. 500.

The quenching effect of compounds on the phosphorescence of riboflavin was tested by adding them to a $2 \cdot 10^{-5}\,M$ aqueous solution of riboflavin and visually observing the phosphorescence in u.v. light after freezing (light source, Philips lamp H.P.W. 125)[16].

Besides the compounds mentioned in Table I it was found that all paramagnetic ions tested, and also oxygen, diminish the photodynamic pimaricin decomposition.

TABLE I

EFFECT OF VARIOUS SUBSTANCES ON PHOTODYNAMIC DESTRUCTION OF PIMARICIN, AND ON RIBOFLAVIN PHOSPHORESCENCE

Added substance	*Photodynamic destruction of pimaricin*		*Riboflavin phosphorescence*	
	Concn.	*% loss pimaricin*	*Concn.*	*Colour of frozen part of the solution*
—	—	23	—	Orange
Cystine	$10^{-3}\,M$	35	$10^{-3}\,M$	Orange
Cysteine	$10^{-3}\,M$	33	$10^{-3}\,M$	Orange
Methionine	$10^{-3}\,M$	21	$10^{-3}\,M$	Orange
Thioglycolic acid	$10^{-3}\,M$	79	$10^{-3}\,M$	Yellow
Glutathione	$10^{-3}\,M$	58	$10^{-3}\,M$	Yellow
Na_2SO_3	$10^{-3}\,M$	93	$10^{-3}\,M$	Orange
Ethyl iodide	$10^{-3}\,M$	22	$10^{-3}\,M$	Orange
Monoiodoacetic acid	$10^{-3}\,M$	23	$10^{-3}\,M$	Orange
KI	$10^{-3}\,M$	2	$10^{-3}\,M$	No colour
KI	$10^{-4}\,M$	5	$10^{-4}\,M$	No colour
Potassium rhodanide	$10^{-3}\,M$	5	$10^{-3}\,M$	No colour
Thiourea	$10^{-3}\,M$	5	$10^{-3}\,M$	No colour
Thiouracil	$10^{-4}\,M$	8	$10^{-4}\,M$	No colour
Ascorbic acid	$5 \cdot 10^{-5}\,M$	Inhibition[1]	$5 \cdot 10^{-5}\,M$	No colour
Hydroquinone	$5 \cdot 10^{-5}\,M$		$5 \cdot 10^{-5}\,M$	No colour
Methanol	75 vol. %	2	5 vol. %	Yellow
Ethanol	96 vol. %	0	5 vol. %	Yellow
Pimaricin*	—	—	$2 \cdot 10^{-5}\,M$	Weakly orange

* On account of the low solubility of the pimaricin a $2 \cdot 10^{-6}\,M$ solution of riboflavin was used to observe the phosphorescence quenching.

These observations are apparently in complete agreement with the paramagnetic quenching of the triplet state[14] (the oxygen molecule is also paramagnetic). The hypothesis of the triplet–triplet transfer as an explanation for the photodynamic reaction described is also strongly supported by the discovery of the quenching of the riboflavin phosphorescence by pimaricin itself.

In summary, a triplet–triplet transfer is proposed as a mechanism of the photodynamic destruction of the polyene antibiotic pimaricin in the presence of riboflavin. The inhibiting effect of added substances on this destruction runs completely parallel to the quenching of the riboflavin phosphorescence by these compounds.

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Uptake of bacitracin by sporangia and its incorporation into the spores of *bacillus licheniformis*

Previous studies[1,2] have suggested a possible relation between the production of the polypeptide antibiotic, bacitracin, and the spore-forming metabolism of *Bacillus licheniformis*. Under a number of different physiological conditions, it has been shown that bacitracin is produced only under cultural conditions that permit eventual spore formation. Thus addition of ethyl malonate to a growing culture inhibits both bacitracin production and spore formation.

While investigating the incorporation of D-[^{3}H]phenylalanine and DL-[^{3}H]-ornithine into bacitracin by whole cells of *B. licheniformis*, we observed that some of the label appeared in the spores. In these experiments the efficiency of incorporation of label into the spores was the same as that into bacitracin, *e.g.* about 1 %. These observations prompted a consideration of the possibility that the bacitracin molecule is incorporated into the spore and thus comprises part of the spore coat[3].

The possibility that bacitracin is incorporated into spores without prior degradation can be tested by using a double label in the molecule. The isotope ratio of the spore should be the same as that of the bacitracin used as the source of label.

One liter of cells was grown in the presence of D-[^{14}C]isoleucine and DL-[^{3}H]-ornithine and the bacitracin that was formed was isolated and purified extensively. An isotope ratio of 2.30 ± 0.5 : 1 (^{14}C:^{3}H) for the bacitracin was determined. At various times, aliquots of cells were removed from a growing culture, harvested, washed, and resuspended in 0.03 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.4, containing 0.03 *M* Ca^{++} and 0.03 *M* Mn^{++}. The doubly labeled bacitracin was added together with a 100-fold excess of unlabeled L-isoleucine and DL-ornithine, and the flasks were incubated with shaking until each was 48 h old when all the cultures were fully sporulated. The spores were harvested, washed four times with distilled water, and the ^{14}C and ^{3}H were measured in a liquid scintillation spectrometer. The results are presented in Table I.

TABLE I

INCORPORATION OF BACITRACIN INTO SPORES

[^{14}C, ^{3}H]Bacitracin (12,700 counts/min from ^{14}C) at an isotope ratio of 2.30:1 (^{14}C:^{3}H) was added to washed cells or spores at the indicated times. Incorporation was under endotrophic conditions.

Age of culture (h)	*Ratio of ^{14}C:^{3}H*	*Efficiency of incorporation (%)*	
		^{14}C	*^{3}H*
12	2.01	9.8	10.2
16	2.29	13.1	13.05
19	2.51	15.5	14.1
22	3.41	23.0	15.4
25	1.66	7.6	10.5
28	1.32	5.3	9.25
31	1.12	3.2	6.6
40	0.99	0.5	1.2

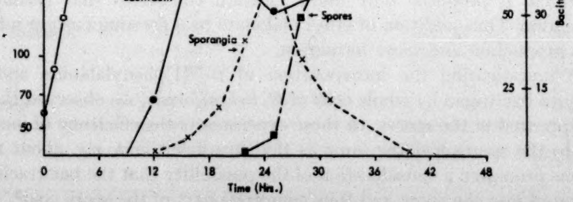

Fig. 1. Time course of formation of bacitracin, sporangia, and spores in a growing culture of *B. licheniformis*.

The maximum incorporation of bacitracin into the spores that are isolated at 48 h occurs between the 16th and the 25th hour of the age of the culture. The isotope ratio of the label in the spores is quite close to that of the bacitracin that was added. An isotope ratio close to 2.30 ± 0.50 : 1 is obtained when the bacitracin is incubated with cultures 12, 16, 19, or 22 h old. The quantity of incorporation is also greatest in these cultures.

When these data are compared with those of the distribution of cells, sporangia, and spores in the culture (Fig. 1), it is obvious that bacitracin is taken into the cell during the period of active sporangia formation. After 70 % of the cells have formed sporangia (25 h), a definite drop in the efficiency of incorporation of bacitracin into spores can be observed.

The spores from the 16-, 19-, and 22-h cultures were pooled, and aliquots were washed on a Millipore filter. No significant decrease in total counts/min was observed when an aliquot of these spores was washed with 5 % trichloroacetic acid, hot 5 % trichloroacetic acid, ethanol, hot ethanol, 8 % urea, 1 *M* phosphate buffer, pH 6.6, or 1 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.8.

These data clearly suggest that the polypeptide antibiotic, bacitracin, is a component of the spore of *B. licheniformis*. It is possible that it functions in a structural manner, since D-amino acid-containing peptides have been reported in the coats of spores of *B. subtilis*, *B. megaterium*, and *B. cereus*[3]. Since the isotope ratio of the spore is the same as that of the bacitracin that was added to the culture, apparently the intact molecule is incorporated directly. This reaction is quite significant quantitatively, with up to 40 % of the label added being incorporated in several experiments. Also the process occurs at the same time that the sporangia are being formed inside the cells, indicating that this is an active process rather than one of occlusion or binding. The latter possibility is not very likely since the label could not be washed off the spores. We do know, however, that the polypeptide antibiotics bind to lipoproteins rather tightly and that this nonphysiological phenomenon could explain the foregoing data[4].

The theory that bacitracin is actually synthesized by the cell as a spore coat component is inviting, but further work is required before a more positive conclusion can be drawn.

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Preliminary Notes

Influence des hormones thyroïdiennes sur la teneur en adenosine triphosphate du foie du rat

Le niveau de plusieurs systèmes enzymatiques à phosphate de pyridoxal du foie du rat est sous l'influence des hormones thyroïdiennes: on observe une diminution de la teneur de ces enzymes chez l'animal thyrotoxique[1-7] tandis que chez l'animal thyroïdectomisé certains de ces enzymes sont présents en quantité plus élevée[6,7]. De plus, on a constaté que le taux de PLP dans le foie de ces animaux varie dans le même sens que les niveaux des activités enzymatiques[8]. Il nous a paru intéressant d'étudier les facteurs responsables de ces modifications, et notamment de rechercher si le taux d'ATP dans le foie change avec l'état thyroïdien de l'animal, ce qui pourrait en partie expliquer des variations des phosphorylations, en particulier de la phosphorylation du pyridoxal.

Dans le présent travail, nous avons pu mettre en évidence une forte diminution du taux de l'ATP dans le foie du rat mâle thyrotoxique par rapport au taux du foie du rat mâle normal, alors que dans le foie de la femelle thyroïdectomisée on n'observe pas de variation significative du taux de l'ATP bien que la quantité totale de l'ATP dans le foie diminue. Les traitements des animaux (injections de thyroxine, thyroïdectomie) ont déjà été indiqués[5,6]. L'ATP est dosé dans un extrait trichloracétique de

TABLEAU I

DOSAGES DE l'ATP DANS LE FOIE DU RAT NORMAL, DU RAT THYROTOXIQUE ET DU RAT THYROIDECTOMISÉ

Les injections quotidiennes de 150 μg de DL-thyroxine sont effectuées pendant 15 jours avant les dosages (Expt. I); la thyroïdectomie a lieu 3 semaines avant les dosages (Expt. II).

	Expt. I (Rats mâles)		*Expt. II (rats femelles)*	
	Témoins	*Injectés*	*Témoins*	*Thyroïdect.*
Nombre de rats	15	15	15	15
Poids des rats (g)				
début des traitements	95 ± 16[*]	94 ± 13	102 ± 18	99 ± 17
fin des traitements	149 ± 16	134 ± 13	150 ± 9	111 ± 14
Poids des foies (g)	6.50 ± 0.29[**]	6.45 ± 0.23	6.20 ± 0.18	3.90 ± 0.12
Taux ATP (μg ATP/g foie frais)	686 ± 65	207 ± 12	995 ± 123	884 ± 88
	$P \ll 0.001$		$0.3 < P < 0.5$	
Quantité totale de l'ATP (μg)	4.450	1.335	6.150	3.450

[*] Moyenne arithmétique des écarts.
[**] déviation standard de la moyenne.
P: facteur de probabilité, par rapport aux animaux témoins.

Abbréviations: ATP, adénosine triphosphate; PLP, phosphate de pyridoxal.

foie après élimination de l'acide trichloracétique par l'éther à 0°; la méthode employée est celle décrite par Strehler et Totter[9], fondée sur la luminescence de préparations d'organes lumineux de *Photinus pyralis* proportionnelle à l'ATP présent. Les résultats obtenus, ont été résumés dans le Tableau I.

Chez le rat mâle, les injections de thyroxine provoquent une forte diminution du taux de l'ATP (de l'ordre de 70 %) sans modifier le poids du foie; chez la femelle, la thyroïdectomie entraîne une forte diminution du poids du foie sans abaisser significativement le taux de l'ATP. De plus, on observe chez la femelle normale un taux de l'ATP plus élevé que chez le mâle normal (30 % en plus; $P \ll 0.001$); il n'est pas possible actuellement d'expliquer ce phénomène. Les résultats obtenus à propos de l'influence des hormones thyroïdiennes sont en accord avec une observation de Vogel[10] sur les érythrocytes de patients hyperthyroïdiens, et avec les effets constatés, sous l'influence de la thyroxine, dans le cerveau du rat par Milcu *et al.*[11] et dans le coeur par Bertolini *et al.*[12]. Signalons cependant que Chilson et Sacks[13] n'ont pu mettre en évidence des modifications significatives du taux de l'ATP dans le foie du cobaye thyrotoxique. Les variations du taux de l'ATP selon l'état thyroïdien de l'animal concordent avec les observations faites précédemment sur le taux de PLP[8] et les niveaux d'activité des enzymes à PLP dans le foie[5-7].

Ces résultats feront l'objet d'une publication ultérieure plus détaillée.

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Discrepancy between thienylalanine activation and protein synthesis in bacteria

Amino acid-activating enzymes are regarded as specific for those amino acids whose chemical structure and steric configuration render them capable of becoming building blocks of proteins. The correlation between amino acid activation and utilization for protein synthesis obtains even for certain unnatural amino acid analogs. It was

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; RNA, ribonucleic acid.

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pointed out by SHARON AND LIPMANN[1], for example, that certain tryptophan analogs which were activated by a mammalian enzyme[1] were also utilized for net protein synthesis in bacteria[2]. Such a correlation should be demonstrable with stronger reason when analog activation and utilization for net protein synthesis are parameters of one and the same organism.

We have found recently, however, that β-2-thienylalanine was strongly activated by an enzyme extract from the phenylalanineless strain C-2 of *Escherichia coli* but unexpectedly was not utilized for appreciable net protein synthesis by this organism.

A bacterial enzyme extract was prepared by grinding 25–50 g of washed wet *E. coli* C-2 with alumina, extraction with 0.05 M Tris buffer (pH 7.2), differential centrifugation, exhaustive dialysis of the $92{,}000 \times g$ supernatant, followed by lyophilization and reconstitution of the solid in a small volume of Tris buffer. Amino acid activation by this enzyme extract was assayed by measuring hydroxamic acid formation after the method of DAVIE *et al.*[3] Phenylalanine hydroxamic acid to be used as a colorimetric standard was prepared by a modification of the procedure of CUNNINGHAM *et al.*[4]. Under the conditions of the assay 1.0 µmole of phenylalanine hydroxamic acid yielded an absorbancy of 0.20, read in a 10 mm cuvette of a Beckman Model DU spectrophotometer at 500 mµ.

The bacterial-enzyme extract activated both phenylalanine and thienylalanine as depicted in Fig. 1. The activation was ATP-dependent; control experiments with heat-inactivated enzyme preparations produced no formation of hydroxamic acid. Activations of thienylalanine and selected amino acids were additive. This is illustrated with methionine by data listed in Table I. In contrast, activation of thienylalanine and phenylalanine was non-additive (Table I), suggesting that both amino acids competed for the same activating enzyme.

The intact bacteria did not form appreciable quantities of protein when thienylalanine was substituted for phenylalanine (Table II). No RNA was synthesized by thienylalanine-supplied *E. coli* C-2.

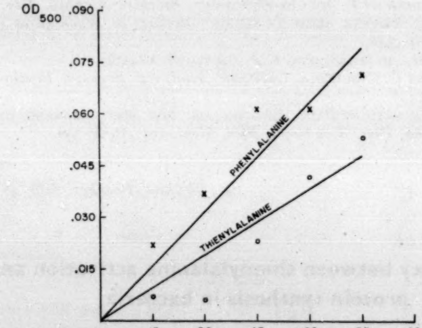

Fig. 1. Hydroxamate formation catalyzed by an enzyme extract from *E. coli* C-2. The assay procedure was that of DAVIE *et al.*[3]. The diagram shows the typical ratio between the reaction rates for phenylalanine and thienylalanine hydroxamic acid formation as observed in numerous experiments. Linearity of the reaction with time was established separately. The enzyme extract exhibited strong pyrophosphatase activity.

TABLE I

ADDITIVITY OF THIENYLALANINE AND METHIONINE ACTIVATIONS AND NON-ADDITIVITY OF THIENYLALANINE AND PHENYLALANINE ACTIVATION

Experimental mixtures contained 1 mmole NH_2OH, 30 μmoles Tris, pH 7.2, 10 μmoles $MgCl_2$, and 10 μmoles ATP in addition to the amino acids listed below in a total volume of 1.1 ml. Incubation was carried out for 60 min at 37°. DL-thienylalanine concentrations were twice those of natural L-amino acids in allowance for the racemic composition of the analog preparation.

Compound activated	*Concentration (mM)*	*Hydroxamate formed (absorbancy at 500 mμ)*
DL-Thienylalanine	17.4	0.121
L-Methionine	8.7	0.163
L-Methionine	17.4	0.185
DL-Thienylalanine +	17.4	
L-Methionine	8.7	0.284
DL-Thienylalanine	14.6	0.095
L-Phenylalanine	7.3	0.121
L-Phenylalanine	14.6	0.155
DL-Thienylalanine +	14.6	
L-Phenylalanine	7.3	0.134

TABLE II

PROTEIN SYNTHESIS IN *E. coli* C-2

Exponentially growing cultures were washed exhaustively and resuspended in fresh glucose-mineral medium with the additions indicated below. Samples were taken at time intervals, the collected bacteria were extracted with hot trichloroacetic acid, and the residues were solubilized in 0.1 *N* NaOH and analyzed for nitrogen by H_2SO_4–H_2O_2 digestion followed by Nesslerization.

Time (min)	*Protein (per cent of initial values)*		
	no phenylalanine nor analog	*50 μg/ml DL-thienylalanine*	*10 μg/ml L-phenylalanine*
0	100	100	100
60	83	107	196
120	100	107	346
180	100	107	—

The inability of thienylalanine to support significant protein and RNA net biosynthesis contrasts markedly with the rate and extent of activation of this analog by the bacterial enzyme extract. This discrepancy constitutes the essential subject of the present communication, and will also be the subject of future communications.

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The biosynthesis of trimethylamine-N-oxide

Despite the widespread occurrence of trimethylamine-N-oxide[1], its biosynthesis has received little attention. MULLER AND IMMENDORFER[2] were unable to demonstrate the oxidation of TMA to TMAO using perfused liver, liver extract or minced liver, although they and others[3-5] obtained evidence favoring the change *in vivo*. In the present work, evidence is presented indicating that this oxidation occurs in liver homogenates. Rat, hog and dogfish liver homogenates, prepared according to the method of FRANTZ AND BUCHER[6], were shown to effect this conversion. For reasons of convenience, hog liver was used in the initial enzymological study, the results of which are reported below.

[^{14}C]TMA·HCl was synthesized from H·^{14}CHO (obtained from Research Specialties Co.) by the method of SOMMELET AND FERRAND[7]. For the preparation of hog-liver homogenates and subcellular fractions, the method of TCHEN AND BLOCH[8] was employed.

The clear solutions resulting from the deproteinization and deionization of incubated samples (for conditions, see Table I) were plated under alkaline conditions prior to counting. Thus, advantage was taken of the volatility of TMA to remove it from the non-volatile reaction product, TMAO.

From Expt. 1 (Table I) it is evident that a crude hog-liver homogenate catalyzes the oxidation of TMA. That the reaction is an enzymic one is indicated by the lack of activity in a boiled homogenate. The activity was retained, and even enhanced, by the removal of mitochondria. Microsomes and supernatant alone exhibited negligible synthesis of TMAO; thus it is clear that both fractions are necessary for this oxidation. To investigate the likelihood of the supernatant serving merely as a source of cofactors, dialyzed and boiled supernatant were separately added back to the microsomes. Little activity was restored by dialyzed supernatant, whilst the boiled fraction was much more effective. In the presence of both, the activity was completely restored. Therefore, Expt. 2 was designed to establish the identity of the necessary cofactor(s). Little stimulation of activity was caused by DPNH or TPN additions, but TPNH or a TPNH-generating system were highly efficient. Thus, the latter two can substitute for the supernatant in the TMAO-synthesizing system.

A requirement for oxygen was demonstrated by incubating the reaction mixture in a helium atmosphere and finding a large decrease in activity. Therefore, it is likely that the oxygen atom of TMAO is derived from molecular oxygen and not from water. MULLER AND IMMENDORFER[2] have suggested that TMA is oxidized via a dehydrogenation reaction, whereby the oxygen atom of TMAO is derived from the aqueous medium. Experiments with ^{18}O are in progress to establish which mechanism is operating.

On the basis of these findings, it is considered likely that TMA is related to the large group of metabolic intermediates which appear to be oxidized by OH^+ or its equivalent[9]. Microsomes, TPNH, nicotinamide and molecular oxygen are commonly implicated in such enzyme systems. When high levels of TPNH are used in our system (as reported in Table I), no stimulation of activity is obtained by the addition of nicotinamide.

Abbreviations: TMA·HCl, trimethylamine hydrochloride; TMAO, trimethylamine-N-oxide; DPNH, reduced diphosphopyridine nucleotide; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; G-6-P, glucose 6-phosphate.

TABLE I

THE FORMATION OF TRIMETHYLAMINE-N-OXIDE FROM TRIMETHYLAMINE BY HOG-LIVER FRACTIONS

		TMAO formed (counts/min)
Expt. 1*	*Substrate*: 0.04 μmole [^{14}C]TMA·HCL (1.42·10^5 counts/min/μmole)	
	Homogenate	1728
	Homogenate (boiled for 10 min)	17
	Homogenate — mitochondria	2479
	Supernatant	50
	Microsomes	80
	Microsomes + supernatant	1053
	Microsomes + dialysed supernatant	156
	Microsomes + boiled supernatant	848
	Microsomes + boiled + dialysed supernatant	1097
Expt. 2**	*Substrate*: 1.37 μmoles [^{14}C]TMA·HCl (5.26·10^3 counts/min/μmole)	
	Microsomes	17
	Microsomes + DPNH	134
	Microsomes + TPN	139
	Microsomes + TPNH	626
	Microsomes + TPN + G-6-P + G-6-P dehydrogenase	1177
	Microsomes + TPN + G-6-P + G-6-P dehydrogenase–O_2	235

The reaction mixture was made up to a total volume of 1 ml with 0.1 *M* phosphate buffer (pH 7.5), and incubated at 37° for 3 h in open tubes. Mixtures were deproteinized with 0.1 ml 50% trichloroacetic acid, deionized with 1 ml Amberlite CG45 (OH^-) suspension (200 mg/ml water), centrifuged and aliquots plated under alkaline conditions prior to counting.

* The various fractions were used in quantities equivalent to their concentrations in the whole homogenate.

** Amounts of cofactors used were: 1.36 μmoles DPNH, 1.23 μmoles TPNH, 1.23 μmoles TPN, 25.4 μmoles G-6-P, and 100 μg of G-6-P dehydrogenase.

To ensure that the compound formed as a result of incubating [^{14}C]TMA·HCl with microsomes and TPNH is TMAO, samples of this radioactive product were co-chromatographed with authentic TMAO in *n*-butanol–formic acid–water (77:10:13, v/v/v). Spots located autoradiographically and colorimetrically[10] were coincident. Identical migration behavior was likewise found by paper electrophoresis in 0.1 *M* sodium acetate buffer (pH 5.0) (12 V/cm for 2 h). After reduction with $TiCl_3$, the radioactive product co-chromatographed with TMA. This evidence strongly supports the identification of the reaction product as TMAO.

CHAYKIN AND BLOCH[11] have reported the formation *in vivo* by rats of nicotinamide-N-oxide from injected nicotinamide. FISH *et al.*[12] have briefly reported the conversion of N,N-dimethyltryptamine and hordenine to their N-oxides by mouse-liver homogenates, but no detailed assessment of the need for cofactors was given, so a comparison with the TMA-oxidizing system is not possible. In view of the experimentally demonstrated biosynthesis of several N-oxides, it is considered possible that a general mechanism exists for their formation. This possibility is being investigated.

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Isolation and identification of the irradiation product of thymine

We have described the rapid conversion by u.v. radiation of thymine in a frozen aqueous solution into a low-absorbing substance[1]. This transfer is reversible since renewed irradiation of the thawed solution of the reaction product leads again to thymine. The irradiation product of thymine has now been isolated and a suggestion for the structure can be made.

Since thymine shows a very high molecular extinction, only dilute solutions can be used in the irradiation procedure (400 mg/l). After freezing in large Petri-discs (35-ml portions) the thymine solution was subjected to u.v. (2537 Å). The solutions were allowed to thaw and the irradiation product was freed from thymine by a repeated extraction with absolute ethanol. In contrast to thymine, the product is almost insoluble in this solvent. The completeness of the extraction was determined by measuring the extinction of the extract. After complete removal of thymine (no residual absorption at 264 mμ) the residue was recrystallized from water (small needles, decomp. 320°). The elementary analysis agrees completely with that of thymine. (Found: C, 47.57, 47.60; H, 4.94, 4.81; N, 22.21, 22.09. Calc. for $C_5H_6O_2N_2$: C, 47.62; H, 4.80; N, 22.21). The substance therefore must be an isomer or a polymer of thymine.

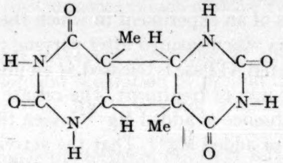
Molecular-weight determination by the usual methods was impossible on account of the low solubility in organic solvents and in camphor and similar substances. For this reason the method of SIGNER (isothermic distillation)[2] was used. The apparatus was made according to CLARK[3]. In order to obtain a sufficiently high vapour pressure of the solvent (water), the temperature of the surrounding water bath was kept at 85°. Even at this temperature the concentration could not be higher than 0.01 *M*. From the results obtained in this way we may conclude upon a dimer.

This assumption has been supported by a crystallographic investigation. The irradiation product crystallizes in the rhombic system and the elementary cell contains 16 molecules of a monomer size. Since this number in a rhombic crystal is usually 8, a dimer is probable.

A third indication for a dimeric structure is found in the formation of a thymine–uracil addition product upon irradiation of a mixture of thymine and uracil in frozen solution.

From mass-spectrographic results, a molecular weight of 126 (monomer) was found. It may, however, be assumed that the molecule is very susceptible to the electron bombardment to which it is subjected in the method and will be immediately split in two thymine molecules. As already mentioned above, the same results can also be obtained by u.v. irradiation of a solution of the irradiation product in water[4].

The i.r. spectrum contains the small peaks of a cyclobutane ring system (about 870 cm^{-1}). In view of the high chemical stability , a C–C bond formation is plausible. The absence of an u.v. absorption in the neighbourhood of 260 mμ and the improbability of other possibilities suggest the following structure



Similar dimerisations are known in the literature[5]. The other dimer with both methyl groups at adjacent C atoms is less probable on account of nuclear-magnetic-resonance analysis. No spin–spin coupling of the two neighbouring H atoms at the cyclobutane ring can be observed. This does not mean that we will rule out or deny the existence of a dimer of this structure. Under special conditions (*e.g.* in deoxyribonucleic acid) its formation might be possible.

This investigation was supported by the U.S. Army. We wish to express our gratitude to Dr. J. BOLDINGH, director of the Unilever Research Laboratory, Vlaardingen, for giving us the opportunity to have the nuclear magnetic resonance and mass-spectrographic analyses done. These analyses were performed by Ir. R. KEUNING and Ir. W. SONNEVELD, whom we thank for their great interest and help. We should also like to thank Ir. J. H. PALM for the crystallographic investigation and Mr. M VAN LEEUWEN for the elementary analysis.

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The release of adenosinetriphosphatases from rat-liver mitochondria during treatment with sound

We have previously reported evidence[1] which indicated that ATP is enzymically hydrolyzed, with the release of P_i, in the presence of intact, isolated rat-liver mitochondria by at least two pathways, one of which was sensitive, the other relatively insensitive, to far-ultraviolet light. The u.v.-sensitive pathway was the one activated by DNP while the u.v.-insensitive pathway was the one activated by Mg^{++} following treatment of the mitochondrial suspension with DOC. We have now been able to prepare a soluble extract of mitochondria, by treatment with sonic vibration, which contains ATPase activities apparently catalyzed by at least two distinct pathways.

Twice-washed rat-liver mitochondria were prepared essentially according to SCHNEIDER AND HOGEBOOM[2] in 0.25 *M* sucrose. The mitochondrial suspension was treated with sound and the resulting suspension centrifuged at 105,000 × *g* (avg.) for between 2 and 4 h. The supernatant was removed and assayed for ATPase activity[1]. Each incubation flask contained soluble extract derived from mitochondria from either 1.6 or 0.8 g of fresh rat liver.

Fig. 1 contains results of an experiment in which the release of ATPase activity from rat-liver mitochondria was measured after varying periods of sound treatment. It is seen that Mg^{++}-supported ATPase is released at an initial rapid rate, approaching an asymptote at about 20 min of treatment. The release curve representing soluble extract incubated in the absence of added Mg^{++} is seen to be of a different character than that in the presence of added Mg^{++}. That the activity in the absence of added Mg^{++} does not represent a mere conversion, by sound, from the Mg^{++}-dependent activity is clear since prolonged treatment in the sonic oscillator results in little or no alteration of activity from the 60-min values. This is also the case when the extract is treated with sound after centrifugation.

Table I contains data concerning the effect of various agents on an extract produced by 15 min of sonic treatment. As described by LÖW[3] for ATPases of intact mitochondria, atebrin is inhibitory as is chlorpromazine. It would thus appear that

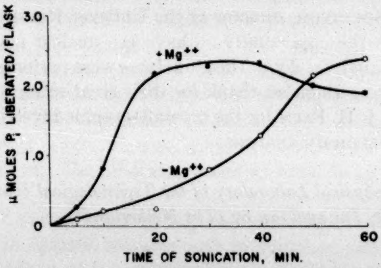

Fig. 1. The release of ATPases during sonic treatment. Each flask contained 100 μmoles Tris–HCl, pH 7.4, 10 μmoles ATP, 4 μmoles $MgCl_2$ when added, and extract derived from mitochondria from 0.8 g of fresh liver. Final vol., 2 ml; gas phase, air; temp., 30°; time of incubation, 20 min.

Abbreviations: ATP, adenosine triphosphate; P_i, inorganic orthophosphate; DNP, 2,4-dinitrophenol; DOC, deoxycholate; FAD, flavin-adenine dinucleotide; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetate.

flavin is involved in the reaction sequence. DPN stimulates ATPase activity in the presence of Mg^{++} and although the stimulation is always slight, it is always present. Other compounds which have been tested for effect and have been found inactive are FAD, vitamin K$_1$, and coenzyme Q$_{10}$-ubiquinone. DNP does not stimulate in the absence of Mg^{++} nor in the presence of 5 m*M* atebrin. The soluble extract displays no ATP-P$_i$ exchange activity.

TABLE I

EFFECT OF VARIOUS AGENTS ON SONIC Mg^{++}–ATPase

Incubation conditions as in Fig. 1.

Additions	*μmoles P$_i$/20 min*
None	2.10
Atebrin, 0.4 m*M*	1.41
Atebrin, 4.0 m*M*	0.50
Amytal, 0.5 m*M*	1.96
Chlorpromazine, 0.5 m*M*	0.43
DNP, 0.1 m*M*	2.42
No Mg^{++}	0.43

FAD (10^{-4} *M*) and FMN (10^{-4} *M*) added to each of above without effect.

Preliminary experiments involving separation of the two types of activity by protamine sulfate and subsequent separation of protein from smaller molecules with sephadex columns indicate that Mg^{++} does not stimulate the Mg^{++}-independent ATPase.

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A TPNH-linked sulfite reductase and its relation to hydroxylamine reductase in enterobacteriaceae

The occurrence of a TPNH-specific hydroxylamine reductase in *Escherichia coli* and related species has been briefly reported[1]. The formation of this enzyme, as gauged

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Abbreviations: TPNH, reduced triphosphopyridine nucleotide; PAPS, 3′-phosphoadenosine-5′-phosphosulfonic acid; DPNH, reduced diphosphopyridine nucleotide; FAD, flavine-adenine dinucleotide.

by the specific activity of cell extracts prepared under standard conditions with the aid of a Nossal oscillator, was strikingly repressed by cysteine and methionine.

The specific nature of the repressive agent suggested that the phenomenon concerned may represent a feedback control relationship[2] reflecting an hithertho unknown, essential role of hydroxylamine reductase in the biosynthesis of sulfur-containing amino acids.

This concept prompted a study designed to explore the enzymic mechanisms underlying the initial stages of sulfate assimilation prior to the attachment of the sulfur atom to the carbon skeleton of serine[3,4]. As revealed by the nutritional date of LAMPEN *et al.*[5] this pathway involves at least two distinct reductive steps:

1. $SO_4^{--} \longrightarrow HSO_3^-$;   2. $HSO^{3-} \longrightarrow H_2S$

Preliminary results seem to indicate that the first step in the above sequence is mediated by a system essentially similar to the TPNH-linked PAPS-reductase described by WILSON AND BANDURSKI in yeast[6] but requiring DPNH rather as the hydrogen donor[7]. The reduction of HSO_3^- to H_2S, on the other hand, was found to be catalyzed by an enzyme specifically linked to TPNH.

As in the case of hydroxylamine reductase, the production of sulfite reductase by the proliferating cells of *E. coli* and other enterobacterial strains proved to be subject to feedback repression by cysteine or cystine and to a lesser extent by methionine; it was not affected, however, by mercaptoacetate or other thiol compounds metabolically unrelated to cysteine. Whereas a mixture of other amino acids showed no effect, addition of serine to the minimal culture medium resulted, depending on the concentration used, in a 2- to 4-fold enhancement of the enzyme yield.

These results appear therefore to bear out the role played by sulfite reductase in the biosynthesis of cysteine from inorganic sulfate and conform with the pertinent data of BOLTON *et al.*[8] obtained by "isotope competition" studies in intact *E. coli* cells.

Hydroxylamine reductase which was determined concomitantly with sulfite reductase followed in all instances a strictly parallel pattern of quantitative response to the various metabolites examined (see Table I).

The specific activity of sulfite reductase was roughly one tenth of that exhibited by hydroxylamine reductase when measured by the rate of TPNH oxidation at their different pH optima (about pH 8 and pH 9 respectively). As shown in Table I, however, notwithstanding this disparity in catalytic potency, the ratio of the two enzymic activities in all the preparations tested varied within a rather narrow range, not exceeding the limits of experimental error inherent in the necessarily different conditions of the assay procedure.

This ratio remained practically unaltered throughout a purification procedure comprising 6 separate steps and resulting in more than a 150-fold increase in the specific activities of the two reductases, as compared with those present in the crude extracts. Furthermore, the catalytic function of the purified preparation with regard to reduction of both sulfite and hydroxylamine exhibited a marked dependence on added FAD. On the other hand, both activities decreased in nearly the same proportion, following various adverse treatments such as mild heating or exposure to low pH. The two reactions also shared a pronounced susceptibility to inhibition by KCN.

The outlined experimental results seem to favor the tentative conclusion that the reduction of sulfite and of hydroxylamine are both catalyzed by a single enzymic

TABLE I

COMPARISON OF HYDROXYLAMINE REDUCTASE AND SULFITE REDUCTASE ACTIVITIES IN EXTRACTS PREPARED FROM *E. coli* CELLS GROWN IN THE PRESENCE OF VARIOUS AMINO ACIDS

Compounds added to minimal medium	*Units/mg protein*		*Ratio a/b*
	Hydroxylamine reductase (a)	*Sulfite reductase (b)*	
None	61.5	5.6	10.8
L-serine (10^{-3} *M*)	107.0	9.7	10.9
L-serine ($2 \cdot 10^{-3}$ *M*)	200.0	17.7	11.3
L-serine ($3 \cdot 10^{-3}$ *M*)	260.0	23.3	11.2
L-cysteine ($2.5 \cdot 10^{-4}$ *M*)	traces	0	—
L-cysteine ($1.5 \cdot 10^{-4}$ *M*)	20.8	2.0	10.4
L-methionine ($2.5 \cdot 10^{-4}$ *M*)	28.4	2.3	12.3
Sodium mercaptoacetate (10^{-3} *M*)	56.9	4.9	11.7

Reaction mixture for assaying hydroxylamine reductase: 2-amino-2-methyl-1,3-propanediol buffer, pH 9, 200 μmoles; $NH_2OH \cdot HCl$ (adjusted to pH 9), 50 μmoles; enzyme (suitably diluted), 0.1 ml. Reaction mixture for assaying sulfite reductase: phosphate buffer, pH 8, 200 μmoles; Na_2SO_3, 0.5 μmole; enzyme, 0.1 ml. The volumes of the reaction mixtures were made up with distilled water to 0.95 ml. The assays were started by addition of 0.1 μmole TPNH in 0.05 ml and the rate of TPNH oxidation was followed by the decrease of absorbancy at 340 mμ during the initial 3–5 min. The values were corrected for "TPNH oxidase" which was concurrently determined in the corresponding reaction mixtures by omitting the respective substrate. A *unit* of activity is defined as the amount of enzyme producing under the above indicated conditions a 0.001 decrease in absorbancy within 1 min.

system. This conclusion is strengthened by the additional observation that sulfite in low concentrations blocked the reduction of hydroxylamine. This apparently competitive relationship is consistent with the fact that the K_m for sulfite was found to be more than hundredfold lower than the corresponding value for hydroxylamine. Moreover, the striking difference in the enzyme affinity for the respective substrates is compatible with the interpretation that sulfite reduction represents the "true" *physiological* function, whereas the reduction of hydroxylamine constitutes an *incidental* capacity of the same enzyme molecule. This concept offers a plausible solution for the puzzling problem regarding the metabolic role of hydroxylamine and its reductase in the organisms studied.

It may be added as a concluding remark that the curious substrate specificity of this enzyme for sulfite and hydroxylamine as well as the inhibition of its catalytic function by cyanide, can be accounted for by postulating a free carbonyl group as the "active site" of the system. Experiments designed to test the validity of this hypothesis are now in progress.

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Uptake of 5-hydroxytryptophan by rat brain

The possible implication of 5-hydroxytryptamine (serotonin) in neurophysiology[1,2] logically leads to an investigation of the uptake of its precursor, 5-hydroxytryptophan, by brain cells. Such a study is indicated also because of recent evidence[3] that the brain does not form 5-HTP, but depends rather on extra-neural sources probably gastro-intestinal mucosa[3,4] for this amino acid.

Studies of the uptake of [1-^{14}C]DL-5-HTP by slices of rat brain and by other tissues have been carried out in a modified Rodnight and McIlwain's medium[5] (pH 7.4). The total number of counts appearing in association with the washed tissue was determined. This radioactivity is a reflection of the 5-HTP in the tissue as well as any subsequent metabolites of this amino acid, bearing the labeled carbon.

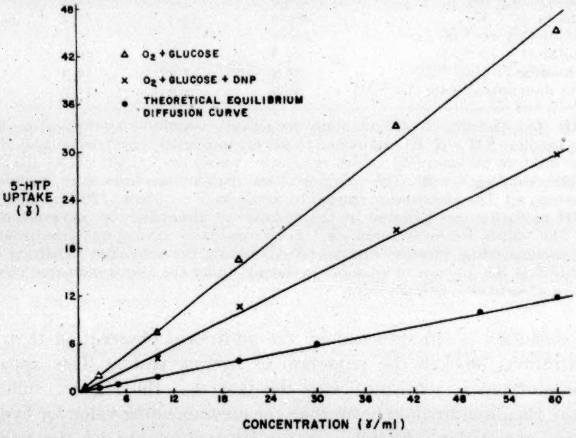

Fig. 1. Relationships of concentration of 5-HTP to its uptake by slices of rat brain. Slices were made sagitally along the central sulcus so as to include both cortical and sub-cortical areas. 200 mg (wet wt.) of slices were placed in 3.0 ml of the medium containing varying amounts of [1-^{14}C]DL-5-HTP (specific activity, $4.6 \cdot 10^6$ counts/min/mmole), with glucose (10^{-2} *M* final concn.) and dinitrophenol (DNP, 10^{-4} *M* final concn.) as indicated. Incubation at 37° for 60 min was carried out in an atmosphere of 97% O_2 and 3% CO_2. At the end of the incubation period, the slices were removed, washed and extracted with 70% ethanol. Radioactivity in the supernatant fraction was then determined by standard counting techniques.

Figs. 1 and 2 suggest that in brain slices there is a facilitated transport of 5-HTP. This may be deduced (1) by the enhancement of uptake seen with oxygen and glucose, (2) by the interference of dinitrophenol with this enhancement, and (3) by the greatly diminished transport observed at 0°. Preliminary experiments with [1-^{14}C]serotonin have indicated that this amine is not transported in the same way as its precursor by brain slices, nor do brain slices behave like platelets in concentrating serotonin[6]. It is of interest in Fig. 2 that more than one-half of the total uptake of 5-HTP by brain slices occurs within 10 min and that after that time all curves approximate the slope of the theoretical equilibrium diffusion curve.

Abbreviation: 5-HTP, 5-hydroxytryptophan.

A comparative study of the uptake of 5-HTP by a number of selected tissues was also undertaken. Data from such a study are summarized in Table I.

It is clear that the greatest uptake of 5-HTP occurs in those cells which have 5-HTP decarboxylase activity and are capable of storing serotonin (e.g., mast cells and brain). It is of interest that a tissue like spleen, which has no decarboxylase activity, but stores large quantities of serotonin (presumably from destruction of thrombocytes) has a low uptake of 5-HTP almost equivalent to the theoretical amount transported by diffusion.

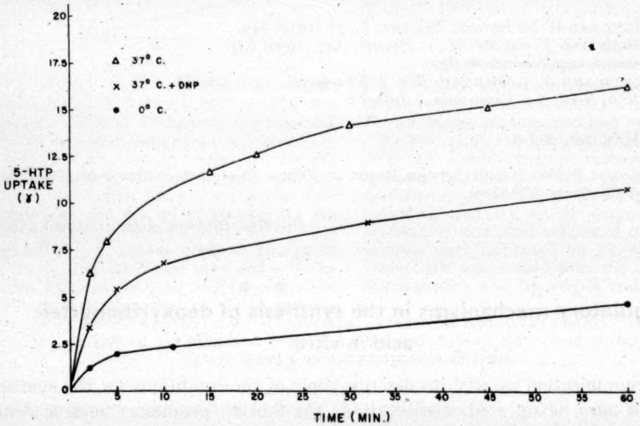

Fig. 2. Rate of uptake of 5-HTP by slices of rat brain. The technique was the same as described in Fig. 1 with the exception that a fixed amount of 20 μg [1-^{14}C]DL-5-HTP was used, and the experiments were run at 37° and at 0°. Glucose (10^{-2} M) was present in all three experiments.

TABLE I

COMPARATIVE UPTAKE OF 5-HYDROXYTRYPTOPHAN BY SEVERAL SELECTED TISSUES

Tissue	*Species*	*5-Hydroxytryptophan decarboxylase activity** [7,8]	*Serotonin storage*** [7,9]	*Uptake of 5-HTP (μg/200 mg)*
Mastocytoma cells	Mouse	+	+	56.73
Brain	Rat	+	+	15.00
Kidney	Rat	+	—	8.74
Liver	Rat	+	—	5.80
Spleen	Rat	—	+	4.39
Ehrlich ascites cells	Mouse	—	—	4.36
			Theoretical equilibrium diffusion value***	4.00

* + indicates activity, — indicates no activity.
** + indicates storage, — indicates no storage.
*** Calculated on the assumption that the tissue is 100 % water, and therefore, represents 0.2 ml.

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Regulatory mechanisms in the synthesis of deoxyribonucleic acid *in vitro*

In this communication we wish to describe some of the conditions for the synthesis of DNA *in vitro*, using a ribonucleotide as the labeled precursor. Such a system should contain the enzymes required for the conversion of the ribonucleotide to the corresponding deoxyribonucleotide, as well as the enzymes required for the incorporation of the latter into DNA. An extract of 5-day old chick embryos obtained by centrifugation of a homogenate at 25,000 × *g* for 45 min was used as a source of the combined enzyme systems.

The conversion of cytidylic acid to deoxycytidylic acid seems to occur at the diphosphate level[1], whereas the deoxyribonucleoside triphosphates[2–4] are the precursors of DNA. Thus, it was expected that the degree of phosphorylation of the ribonucleosides would be critical in defining the best conditions for the overall reaction. Fig. 1 shows that the addition of ATP alone was better than the presence of an ATP-regenerating system in maintaining an optimal balance between di- and triphosphates.

A maximal rate of DNA synthesis from deoxyribonucleotides occurs when all four deoxyribonucleotides are present[2–4]. In the present system, using [^{14}C]deoxyCMP as the labeled precursor, the synthesis of DNA was stimulated by the addition of deoxyATP, deoxyGTP and TTP. In contrast, DNA formation from [^{3}H]CMP was strongly inhibited by the addition of these deoxynucleoside triphosphates. As is shown in Fig. 2, this inhibition occurred during the transformation of the ribosyl to the deoxyribosyl compound. Effective inhibition was observed at deoxyribonu-

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Abbreviations: DNA, deoxyribonucleic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; TTP, thymidine triphosphate; CMP and CTP, cytidine 5′-mono- and tri-phosphate, respectively.

cleotide levels, which were very low when compared with the levels of CMP and ATP. Fig. 2 shows that in the presence of 155 mμmoles CMP and 1 μmole ATP about 3 mμmoles deoxyGTP cause a 50 % inhibition of the reaction.

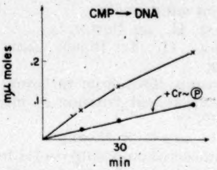

Fig. 1. Energy requirement for the overall conversion of CMP → deoxyCMP → DNA. The incubation mixture contained 114 mμmoles [^{32}P]CMP (4.6·10^6 counts/min), 1.0 μmoles ATP, 5.0 μmoles $MgCl_2$, 100 μg heated calf thymus DNA[5], 15 μmoles tris(hydroxymethyl)aminomethane–HCl buffer, pH 7.4, 0.4 μmole mercaptoethanol, and 0.3 ml of enzyme (14 mg protein/ml) in a final volume of 0.41 ml (×—×); in addition, 4.5 μmoles creatine phosphate and 100 μg creatine kinase were added to the samples indicated by + Cr ~ P (●—●). The samples were incubated at 37°. DNA was extracted and assayed[6] and the results are expressed as mμmoles radioactive precursor incorporated into DNA. Analysis of the extracted [^{32}P]DNA identified deoxy-CMP bound in internucleotide link as the radioactive component of DNA.

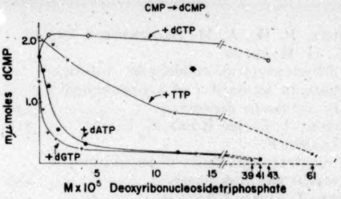

Fig. 2. Effect of deoxyribonucleoside triphosphates on the conversion of CMP to deoxyCMP. The incubation mixture contained 155 mμmoles [^{3}H]CMP (2.85·10^6 counts/min), 1.0 μmole ATP, 15 μmoles tris(hydroxymethyl)aminomethane–HCl buffer, pH 8.0, 0.4 μmole mercaptoethanol, deoxyribonucleoside triphosphates as indicated, and 0.3 ml of enzyme (13 mg protein/ml) in a final volume of 0.38 ml. The samples were incubated for 15 min at 37°. DeoxyCMP was isolated from the acid-soluble fraction after acid hydrolysis and analyzed[7].

In summary, the results indicate that optimal rates of DNA synthesis from CMP were obtained *in vitro* when a critical level of phosphorylated intermediates existed. It has also been shown that the conversion of CMP to deoxyCMP (and indirectly the synthesis of DNA) was strongly inhibited by the presence of deoxyATP, deoxyGTP and to a lesser extent by TTP; in contrast, deoxyCTP showed no such inhibition. These phenomena may be components of a homeostatic mechanism governing the synthesis of DNA.

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